Sickle cell disease, pathophysiology and clinical complications
van Beers, E.J.

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Sickle cell disease, pathophysiology and clinical complications

Ward van Beers
Sickle cell disease, pathophysiology and clinical complications

Eduard Johannes van Beers
Sickle cell disease, pathophysiology and clinical complications. Dissertation, University of Amsterdam, Amsterdam, The Netherlands

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Sickle cell disease, pathophysiology and clinical complications

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Faculteit der Geneeskunde
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Part I:
General overview
and
clinical complications
Pathophysiology and treatment of sickle cell disease
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Abstract

Sickle cell disease is a hereditary haemoglobinopathy caused by a mutation in the \( \beta \)-globin gene. The disease is characterised by recurrent vaso-occlusive crises resulting in severe organ damage and a sharply reduced life expectancy. The formation of haemoglobin-S polymers in hypoxic conditions plays a pivotal role in sickle-cell disease and produces the characteristic phenotype of sickle-shaped erythrocytes that promote vaso-occlusion. Endothelial cell activation, enhanced erythrocyte and leukocyte adhesion, vasoconstriction and coagulation activation play an important role in vaso-occlusive crises.

Treatment of pain and hydration remain the main interventions in the management of vaso-occlusive crises. Hydroxyurea has been shown to prevent vaso-occlusive crises by increasing the amount of foetal haemoglobin. Allogeneic stem-cell transplantation is the only curative therapy. However, transplantation-related mortality, graft-versus-host disease and the limited availability of HLA-identical donors restrict this therapeutic option.
**Introduction**

Sickle cell disease (SCD) is a recessive monogenic inherited disorder affecting the hemoglobin molecule by a mutation in the gene encoding for β-globin. In addition to homozygotic SCD (HbSS, sickle-cell anaemia), SCD also includes double heterozygous states such as hemoglobin SC disease (HbSC), hemoglobin SD or SE disease (HbSD and HbSE) and Hbsβ-thalassemia (HbS/β-thal). The hallmark of SCD is the recurrent episode of severe pain caused by ischemia resulting from vaso-occlusion of bones, the so-called vaso-occlusive crisis or vaso-occlusive crisis. The first signs and symptoms may occur at the age of six months and can lead to frequent hospital admissions for the treatment of vaso-occlusive crises. On the long run, the chronic hemolytic anemia and recurrent micro and macrovascular occlusion result in extensive organ damage and subsequently in a reduced life expectancy. The mean life expectancy for homozygous SCD patients in North America is 42 years for males and 48 years for females.

**Epidemiology**

SCD is a prevalent disease, which originates from Central and West Africa, India and Saudi Arabia. As a result of slavery and migration the disease is present throughout the entire world with an estimated prevalence of sickle cell trait (carriership of HbS) ranging from 8% in the Afro-American population of the United States of America to 40% in endemic areas in West Africa, India and Saudi Arabia. In the Netherlands, subjects with sickle cell trait are found to originate predominantly from Surinam and the Netherlands Antilles but recently an increasing number of HbS-carriers and sickle cell patients are immigrants from West African countries such as Ghana and Nigeria. A recent survey in the Academic Medical Center in Amsterdam among 1016 pregnant women originating from Surinam and West Africa showed a prevalence of sickle cell trait of 12% and 15% respectively. The total number of patients with SCD in the Netherlands is estimated to be at least 600.

**Clinical presentation**

Sickle cell disease is characterized by recurrent vaso-occlusive crises triggered by infection, heavy physical exertion, high altitude, dehydration, exposition to cold or mental stress, but may also develop spontaneously. More than 50% of the patients with SCD experience more than one vaso-occlusive crisis per year for which hospital admission and treatment with opiates is required. However, most episodes of vaso-occlusive crises do not require hospitalization and are treated at home. Chronic hemolytic anemia and recurrent vaso-occlusion of the microvasculature are responsible for the progressive development of disabling organ damage such as, splenic infarction, avascular osteonecrosis, cardiomyopathy, renal failure, leg ulcers and priapism. In particular,
splenic dysfunction is responsible for an increased risk of infection with encapsulated bacteria, such as Streptococcus pneumoniae. Other complications that are associated with a strongly reduced life expectancy are stroke, acute chest syndrome, pulmonary hypertension, and acute splenic sequestration (in children). As much as 10% of children with SCD are diagnosed with acute stroke and in at least 20% of these children signs of cerebral infarction can be found when magnetic resonance imaging (MRI) of the brain is used to screen for this complication. Acute chest syndrome is defined as a syndrome of pulmonary complaints (dyspnea, thoracic pain and fever) in combination with new infiltrate on chest X-ray and is an important cause of death in sickle cell disease. Acute chest syndrome may be caused by airway infection, fat emboli or pulmonary infarction but most cases develop during vaso-occlusive crises without a clear explanation. Recently, it was shown that 30% of adult patients with SCD have pulmonary hypertension (PHT) which is correlated with early death. The pathogenesis of PHT has not fully been elucidated but nitric oxide (NO) scavenging by free circulating hemoglobin due to chronic intra-vascular hemolysis appears to play an important role. Acute splenic sequestration is an acute complication in young sickle cell patients characterized by a rapid enlargement of the spleen due to massive erythrocyte sequestration resulting in a life-threatening anemia.

![Image](image.jpg)

**Figure.** The many sickled erythrocytes are clearly visible when viewed under a microscope. (Peripheral blood smear, Jenner-Giemsa stain, x12,500)
Pathophysiology

The formation of polymers of sickle hemoglobin (HbS) is responsible for the characteristic ‘sickle’ phenotype of erythrocytes in SCD. In contrast to the simple mechanical obstruction of the microvasculature by sickled erythrocytes, it has now become clear now that etiology of vaso-occlusion in sickle cell disease is the result of a complex interplay between endothelial activation, cytokine release, intercellular adhesion, vasoconstriction, and coagulation activation.

Polymerization of HbS

SCD is caused by a single nucleotide substitution in the gene encoding the β-globin protein. This nucleotide substitution results in the production of less water-soluble hemoglobin which polymerizes upon deoxygenation. The polymerization of HbS causes the characteristic phenotype of the sickled erythrocyte. The extent of polymerization is defined not only by deoxygenation but also by the intracellular HbS and fetal hemoglobin (HbF) content. Until birth the β-globin gene is inactive and the γ-globin genes are responsible for the production of HbF. High HbF and low HbS concentrations in the erythrocytes of newborns with SCD result in an almost asymptomatic state during the first six months of life. In comparison, cellular dehydration of erythrocytes increases the HbS concentration and causes increased polymerization of HbS and formation of sickled cells. Cellular dehydration is caused by the loss of intracellular water through cytokine activated Gardos-potassium channels.

Endothelial activation and increased adhesion

Recent observations have revealed that the pathophysiology of SCD is not limited to the occlusion of the microcirculation by the entrapment of sickled cells. A chronic inflammatory reaction characterized by cytokine production, endothelial activation, coagulation activation and transmigration of monocytes constitutes another important pathophysiological mechanism in SCD. Under normal circumstances the duration of the exposure of erythrocytes to hypoxic and acidic conditions in the microvasculature is too short to induce local polymerization of HbS. However, due to local adhesive interactions of erythrocytes, leukocytes and endothelial cells the blood flow velocity in the microvasculature decreases. Subsequently, local polymerization of HbS and formation of sickled cells occur, which ultimately leads to complete vaso-occlusion.

The resulting ischemia is responsible for subsequent inflammatory reactions that cause further activation of the endothelium. Thus, vaso-occlusion in SCD seems to start with adhesion of erythrocytes and leukocytes to adhesion molecules on activated endothelial cells in predominantly post-capillary venules. The role of leukocytes in this cascade is illustrated by the fact that leukocytosis is a risk factor for the onset of the acute chest syndrome and vaso-occlusive crisis in
asymptomatic sickle cell patients. Both during vaso-occlusive crises and asymptomatic periods, sickle cell patients have a high number of circulating endothelial cells with increased expression of adhesion molecules such as ICAM-1, VCAM-1, en P- en E-selectin, reflecting ongoing endothelial activation and damage. In murine models of SCD, P-selectin appeared to play a pivotal role in experimental vaso-occlusion and inhibition of P-selectin either by neutralizing antibodies or the use of P-selectin knock-out mice prevented experimentally induced vaso-occlusion. These observations suggest a prominent role of adhesion molecules, such as P-selectin, in the pathophysiology of SCD.

Vasoconstriction
Nitric oxide (NO), produced by the endothelial cells, has a strong vasodilatory and blood flow regulatory effect. Due to chronic hemolysis, high concentrations of extra-cellular hemoglobin can be found in the circulation of patients with SCD. This free hemoglobin scavenges NO resulting in a compensatory increased production of NO, and subsequently a lower concentration of L-arginine, the substrate for NO-synthase. Increased production of NO during low substrate availability results in the production of superoxides instead of NO. This results in less NO bioavailability and more oxidative stress and endothelial damage caused by the superoxides. Decreased bioavailability of NO will result in vaso-occlusion partly due to an increase in the production of the vasoconstrictor peptide endothelin-1. Hemolysis induced scavenging of NO is an important etiological mechanism in the development of sickle cell related pulmonary hypertension.

Coagulation activation
Patients with SCD have chronically activated coagulation. Factors promoting this hypercoagulable state are increased expression of tissue factor on circulating monocytes and endothelial cells and increased expression of procoagulant phospholipids such as phosphatidylserine on erythrocytes. Coagulation activation may affect vaso-occlusion in sickle cell by numerous mechanisms. Importantly, thrombin is one of the strongest activators of endothelial cells and is responsible for the increased expression of P-selectin on endothelial cells and monocytes in SCD thereby promoting cellular adhesion. Despite this, to date no studies have provided convincing evidence that anti-coagulant treatment may decrease the frequency of vaso-occlusive crises in patients with SCD.
Treatment

Pain
Acute microvascular vaso-occlusion results in ischemia of the involved tissue and induces severe pain. Pain is therefore a very common symptom of sickle cell disease which strongly impairs quality of life of patients with SCD. Personalized treatment protocols of pain consisting of acetaminophen, codeine and NSAIDs, will assist patients in treating mild and intermediate vaso-occlusive crises at home. Unbearable pain is the most frequent reason why patients with SCD visit the emergency department. Usually treatment with intravenous opioids, such as morphine, is necessary. However, despite the powerful analgetic effect of morphine, optimal pain relief is often hampered because of physicians’ fear for addiction and side-effects and lack of objective and clear parameters to assess the severity of the vaso-occlusive crisis. The use of pethidin is discouraged because of the short half-time, toxic metabolites and the increased risk of addiction.

Transfusion therapy
Blood transfusion increases oxygen carrying capacity and decreases HbS percentage and therefore has several specific indications in SCD. (table) However, transfusions have considerable side effects including transfusion related infections, iron overload and allo-immunisation despite the transfusion of Kell and Rhesus C and e matched blood. Because of these complications, the indications for blood transfusion in patients with sickle cell disease are limited. Important indications for exchange transfusion in order to reduce the HbS% <30% are acute neurological complications, severe acute chest syndrome and sepsis with multiorgan failure. Chronic transfusion has shown to be a successful strategy to prevent stroke in sickle cell patients with previous stroke or children at high risk for stroke identified by an increased cerebral blood flow measured by transcranial Doppler. Transfusion up to a hemoglobin level of 6.0-6.5 mmol/l prior to surgical interventions has demonstrated to be as effective in the prevention of postoperative complications as exchange transfusion. Prophylactic transfusion during pregnancy is controversial since no effect on pregnancy outcome was demonstrated despite the reduced incidence of vaso-occlusive crises and acute chest syndrome.20
Table. Indications for transfusion therapy in sickle cell disease.\(^3\)

<table>
<thead>
<tr>
<th>Acute</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Acute anaemia: splenic and/or liver sequestration and severe aplastic crisis</td>
</tr>
<tr>
<td>• Acute stroke</td>
</tr>
<tr>
<td>• Acute Chest Syndrome</td>
</tr>
<tr>
<td>• Preoperative</td>
</tr>
<tr>
<td>• Sepsis</td>
</tr>
<tr>
<td>• Refractory priapism</td>
</tr>
<tr>
<td>• SCD-multi-organ failure syndrome</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chronic</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Prophylaxis against recurrent stroke</td>
</tr>
<tr>
<td>• For stroke prevention when transcranial doppler velocities are abnormal in children</td>
</tr>
<tr>
<td>• (Severe) pulmonary hypertension</td>
</tr>
<tr>
<td>• Refractory congestive heart failure</td>
</tr>
<tr>
<td>• Symptomatic anemia</td>
</tr>
</tbody>
</table>

Screening, prevention, vaccination and antibiotics

Annually 40 to 60 children with SCD are born in the Netherlands and introduction of a nationwide screening program for neonates and primary prevention for SCD has been introduced successfully since January 2007. In other countries, screening programs to diagnose children with SCD at early age have proven to be effective in the prevention of fatal infections due to splenic dysfunction.\(^{20}\) Active immunisation of young children against H. Influenzae and S. Pneumoniae combined with antibiotic prophylaxis until the age of 5 has dramatically decreased mortality among children with SCD.

Hydroxyurea

A landmark randomized controlled trial showed that hydroxyurea reduces the frequency of vaso-occlusive crises in patients with SCD.\(^{22}\) Next to the induction of HbF, hydroxyurea also reduces adhesive interactions between sickle cells and the activated endothelium and seems to improve production of NO. Furthermore, recent reports have shown that hydroxyurea therapy also prevents sickle cell related organ damage.\(^{23}\) Yet, 40 % of patients does not respond to hydroxyurea therapy.\(^{23}\) Furthermore, hydroxyurea may be toxic or oncogenic after long term use despite the fact that so far no increased incidence of leukemia or myelodysplasia has been reported in SCD patients with prolonged use of hydroxyurea. Hypo-methylating agents, such as 5-azacytidine en decitabine (5-azadeoxycytabine), have an even higher potency than hydroxyurea to increase HbF. To date, toxicity and the need for parenteral administration prevent use of these drugs in daily practice.

Anti-inflammatory and anti-adhesive drugs

As discussed earlier, pro-inflammatory stimuli cause endothelial activation and subsequent increased expression of adhesion molecules which in turn induce leukocytes and erythrocyte
adherence to the vascular wall, which ultimately leads to vaso-occlusion. Consequently, inhibition of the inflammation, endothelial activation, or blockade of adhesion molecules could be of value in the treatment of SCD. Sulfasalazine, an anti-inflammatory drug, decreased expression of adhesion molecules on circulating endothelial cells and improved microvascular blood circulation in a murine model of SCD. A small study in humans confirmed these effects of sulfalazine on adhesion molecule expression. Treatment of vaso-occlusive crises in children with SCD with the anti-inflammatory methylprednisolone resulted in a shorter duration of admission and decreased analgesics use. However, in this study many patients were readmitted because of rebound vaso-occlusive crises after withdrawal of the methylprednisolon. Specific interventions that blocking adhesion molecules such as integrin αVβ3 and P-selectin, have only been tested in animal models of SCD but seem promising. Another important factor in the perseverance of chronic inflammation in patients with SCD are oxygen free radicals which are produced during reperfusion of ischemic tissue. Administration of acetylcysteine, a potent antioxidant, to patients with SCD resulted in a decrease of circulating sickled cells and a non-significant decrease in the number of vaso-occlusive crises.

**Stem cell transplantation and gene therapy**

To date, the only available curative treatment option for patients with SCD is allogeneic stem cell transplantation. Despite improved techniques and the development of reduced intensity stem cell transplantation (RIST) without the use of toxic myeloablative agents, treatment related mortality (5-10%) and graft-versus-host disease remain important problems. Given the risks of stem cell transplantation only a subgroup of strictly selected young patients should be considered for this treatment. Another potential curative treatment option that may become available in the future, is gene therapy. However, expectations of gene therapy are low for the near future, as it is still in a very experimental stage of development.
Chapter 1

Conclusion

SCD is a severe systemic disease with a strongly reduced life expectancy. Endothelial dysfunction plays a central role in the pathophysiology of SCD. Although the knowledge about the pathophysiology of SCD has been rapidly growing, only hydroxyurea has been added to the compendium of treatments so far. We expect allogeneic stem cell transplantation to obtain a more prominent role in the treatment of young children with SCD and a high risk of severe organ damage. However, it is important to realize that most SCD patients live in the part of the world with relatively limited medical resources and thus stem cell transplantation will not be a feasible treatment option. For current practice best medical and social support of patients remain vitally important to help them fight against their unpredictable illness and to prevent early complications and death.
This thesis

The aim of the present thesis is three-fold:
1. To gain more information on the prevalence of clinical complications and silent organ damage (chapter 3), to optimize treatment of vaso-occlusive crisis (chapter 2) and to explore potential new endpoints in the assessment of vaso-occlusive crisis (chapter 4). 2. To increase insight into the pathophysiology of SCD on a microvascular level (chapters 5-6). 3. To elucidate the prevalence, clinical presentation and pathophysiology of sickle cell related pulmonary hypertension (chapters 7-11).

In the first part of this thesis, we present the results of a randomized controlled trial to study the use of patient controlled anaesthesia (PCA) versus continuous infusion of morphine for the treatment of the vaso-occlusive crisis in SCD (chapter 2). Next, we present the results of a systematic evaluation of a large cohort of sickle cell patients in order to assess the relation between the frequency of vaso-occlusive crises and sickle cell related organ damage and clinical complications (chapter 3). Thirdly, we evaluated the use of real-time microvascular blood flow measurement with a new intravital microscopic technique of side dark field imaging in patients with SCD during vaso-occlusive crisis and steady state in comparison with healthy controls. (chapter 4)

In the second part of this thesis, the etiological role of microparticles in the procoagulant nature of SCD during vaso-occlusive crisis and steady state was studied in chapter five. In the sixth chapter, we performed a study to assess the volume of the anti-adhesive and anti-coagulant endothelial glycocalyx in both patients with SCD and controls.

In the third part of this thesis, research focuses on pulmonary hypertension. Pulmonary hypertension is a recently recognized complication which occurs in approximately 30% of adult patients with SCD and is associated with an increased risk of early death. In chapter 7, we present a large study in which the role of macrovascular occlusion of large and medium sized pulmonary arteries is evaluated by ventilation/perfusion scanning in a group of consecutive sickle cell patients. In chapters 8 and 9, we further try to elucidate the etiology of pulmonary hypertension in SCD by exploring the role of coagulation activation and plasma levels of inhibitors of nitric oxide-synthase in sickle cell patients with and without PHT Chapter 10 describes discriminative value of an array of diagnostic tests that are frequently used for the evaluation of patients with PHT. In chapter 11, we explored the impact of pulmonary hypertension in patients with SCD on exercise intolerance.

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The authors like to acknowledge the laboratory of hematology for producing the peripheral blood smear and the figure.
References

Pathophysiology and treatment of sickle cell disease


Patient controlled analgesia versus continuous infusion of morphine during vaso-occlusive crisis in sickle cell disease, a randomised controlled trial

(Based on: Am J Hematol. 2007 Nov;82(11):955-60)

Registered trial: ISRCTN74336585

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Abstract

Intravenous morphine is the treatment of choice for severe pain during vaso-occlusive crisis in sickle cell disease (SCD). However, side-effects of morphine may hamper effective treatment and high plasma levels of morphine are associated with severe complications such as acute chest syndrome. Furthermore, adequate dosing remains a problem since no objective measurement of pain severity exists and analgesia should be titrated upon the patient’s reported pain. Patient-controlled analgesia (PCA) may therefore be an interesting alternative since patients can titrate the level of analgesia themselves.

In this randomized controlled study, the efficacy of intravenous morphine administration with PCA was compared with continuous infusion (CI) of morphine in patients with SCD during vaso-occlusive crisis.

Twenty five consecutive episodes of vaso-occlusive crisis in 19 patients with SCD were included in the study. Patients in the PCA-group had a markedly and significant lower mean and cumulative morphine consumption as compared to the patients in the CI-group (0.5 mg/h versus 2.4 mg/h (P<0.001) and 33 mg versus 260 mg (P=0.018) respectively). The mean daily pain scores were comparable (4.9 versus 5.3). The lower mean and cumulative morphine consumption in the PCA-group led to significant less nausea and constipation during treatment as compared to the CI-group (area under the curve respectively 11 versus 18 (P= 0.045) and 30 versus 45 (P= 0.021)). Furthermore, a non-significant reduction in the duration of hospital admission of 3 days was observed in the PCA-group.

Patient controlled analgesia results in adequate pain relief at a much lower morphine consumption and should considered to be first choice in morphine administration to sickle cell patients admitted with vaso-occlusive crisis.
Introduction

Recurrent painful episodes due to vaso-occlusive crises are the hallmark of sickle cell disease (SCD) and the most common cause for hospital admission in these patients. The episodes of severe pain are caused by local vaso-occlusion within the bone marrow leading to bone infarction and the release of inflammatory mediators. The incidence of painful episodes requiring hospital admission is estimated at once a year for the whole group of patients with SCD but may vary highly between and within individual patients. Approximately 5% of the patients with SCD are responsible for more than a third of all hospital admissions with vaso-occlusive crises. Frequent admission with vaso-occlusive crises is an important prognostic factor and has been associated with mortality in SCD. An acute vaso-occlusive crisis is generally treated with hyperhydration and analgesia. Although most episodes of vaso-occlusive crisis in patients with SCD are treated at home with oral analgesics such as acetaminophen and/or nonsteroidal anti-inflammatory drugs (NSAIDs), many patients are eventually admitted for intensive pain treatment requiring intravenous morphine. Despite its good analgesic efficacy, morphine has many dose-related side-effects including nausea, constipation, pruritus, sedation and hypoventilation. Moreover, morphine administration has been related to the development of acute chest syndrome. A post-hoc analysis of a study comparing oral morphine administration with continuous intravenous infusion (CI) of morphine in patients with SCD showed that the incidence of acute chest syndrome was directly related to the plasma levels of morphine and its active metabolites. Therefore, strict regulation of the administered morphine dose is advocated in patients with SCD. In general, there are no objective measurements of pain severity and analgesia has to be titrated upon the patient’s reported pain, preferably with the use of pain-measurement scales to guide the intensive treatment. Patient-controlled analgesia (PCA), which allows patients to take control over the treatment of pain, was successfully introduced in the management of postoperative pain. Although PCA has also been used in patients with SCD, no controlled trials in patients with vaso-occlusive crisis have been performed so far.

The aim of our study was to determine the efficacy of PCA in vaso-occlusive crisis in a prospective randomized controlled trial in patients with SCD. Here we show that, morphine administration with PCA lead to markedly lower morphine consumption than dose-adjusted CI of morphine while both methods resulted in comparable pain relief.
Methods

Subjects
This randomized clinical trial was conducted at the Academical Medical Centre in Amsterdam, the Netherlands. The study was approved by the local medical ethics committee and all participating patients gave written informed consent. Consecutive patients with SCD requiring parenteral analgesia for pain during a vaso-occlusive crisis were considered eligible for the study. Inclusion criteria were: SCD (HbSS, HbSC, HbSβ0 and HbSβ+); the presence of an episode of pain caused by vaso-occlusive crisis necessitating treatment with intravenous morphine; age more than 17 years. An episode of pain caused by vaso-occlusive crisis was defined as the occurrence of pain in the extremities, back, abdomen, chest, or head that led to a clinic visit, and could not be explained except by SCD.29 All patients received a pain flow-chart on the outpatients-clinic. With use of this flow-chart patients self administer pain medication, starting with 500 mg acetaminophen 6 times daily and adding 50 mg diclofenac 3 times daily, when needed. To be admitted for intravenous morphine treatment pain scores had to be more than 4 during at least four hours with maximum self-administered pain medication (500 mg acetaminophen 6 times daily and 50 mg diclofenac 3 times daily). Excluded from the study were patients who already received opioids for more than 24 hours or patients that were allergic or intolerant to morphine.

Study Protocol
Patients were randomized between treatment with intravenous morphine using a PCA-pump (Perfusor® fm, Braun, Melsungen, Germany; PCA-group) or dose-adjusted CI administration of morphine (CI-group). Patients who met the inclusion criteria on a subsequent admission were crossed over to the alternative study arm. Randomization was performed in blocks of six with closed envelopes, containing the designated morphine delivery regimen. Data were analyzed on an intention to treat basis. In both intervention groups, the aim of treatment was to establish an adequate level of pain relief. A pain score of five or less on an 11-point verbal response scale (0 = no pain, 10 = worst pain) was accepted as an adequate level of pain relief.30,31 Pain scores were collected four times a day. To get an estimate of mean pain intensity during treatment all verbal response pain scores during treatment were averaged. The change between a single pain measurement on a visual analogue scale (VAS) at baseline and a single measurement after two days of treatment was used to measure difference in pain relieve (0 mm = no pain, 100 mm = worst pain).

Patients randomized to the PCA-group received a single bolus injection of 5 mg morphine followed by patient-controlled bolus of 0.01 mg/kg. The PCA device allowed patients to self-administer an intravenous bolus of morphine by pressing a button which was attached to their bed.
Maximal one bolus every 5 minutes could be administered (a lockout of 5 minutes). If this dosage did not result in adequate pain relief, the bolus dose was increased to 0.02 mg/kg with a lockout of again 5 minutes. Patients in the PCA-group did not receive any continuous infusion (background infusion) on top of the self-administered boluses of morphine. Patients in the CI-group received a single bolus injection of 5 mg followed by CI of 0.03 mg/kg/h. After pain assessment by the attending nurse the morphine dose was increased when needed with cumulative steps of 1 mg/h until adequate pain relief was obtained or side-effects became intolerable. The continuous morphine dosage was decreased in steps of 1 mg/h if pain scores where five or lower or at the patient’s request.

Next to their designated morphine delivery regimen, all patients received additional oral pain treatment consisting of 500 mg acetaminophen 6 times daily and 50 mg diclofenac 3 times daily during the whole admission. Patients with contra-indications or intolerance for diclofenac received tramadol 50 mg, 3 times daily in combination with acetaminophen.

**Outcome**

The primary outcome of this trial was the cumulative and mean hourly morphine consumption, pain intensity score and cumulative side-effects during treatment with intravenous morphine. Length of hospital stay, duration of treatment, and quality of life were secondary outcomes. Hourly and cumulative daily dose were registered during admission. Pain intensity was assessed and recorded four times a day with a verbal response pain scale on an 11-point scale. To account for variances in verbal response pain scales during vaso-occlusive crisis also the worst and least daily painscore were analyzed.32 Because the experience of pain may vary from person to person in perception, response and reported intensity also individual change in pain intensity, importance of pain control, and perceived pain control were assessed separately from the other measurements between the day of admission and two days later.33 Perceived pain intensity, importance of pain control and perceived control of pain was also assessed with a VAS, with 0 mm designated “not at all important” or “not at all under control” and 100 mm “very much important” or “completely under control”, respectively. In addition to above measurements change in quality of life was assessed between the day of randomization as a baseline measurement and two days later. Quality of life was measured using the Medical Outcomes Study 36-item Short Form Health Survey (SF36).34,35 Scores on the SF-36 items were aggregated into a Physical Health Summary (PHS) and a Mental Health Summary (MHS) to compare physical and mental health QoL outcomes.

**Side-effects and adverse events**

Side-effects of morphine, consisting of nausea, pruritus and sedation, were scored daily on an 11-point scale (0 = no symptoms, 10 = worst symptoms). A day without defecation or the need for a
rectal enema scored 10 points on the constipation-scale. The need for medical intervention for side-effects (anti-emetics, antihistamines) was scored plus 5 points on the appropriate scale. To quantify the cumulative experienced side-effects, the area under the curve (AUC) for the separate side-effect scores during treatment of each individual admission was calculated. Mean oxygen saturation was measured daily with a pulse oximeter (Datascope Accutorr Plus; Datascope Corporation. Paramus, NJ. USA). All adverse events were registered. Acute chest syndrome was defined as a new pulmonary infiltrate on a chest x-ray in the presence of chest pain, temperature >38.5°C, tachypnea, wheezing or cough.36

![Diagram](image)

**Figure 1. Trial profile.**

Patients who met the inclusion criteria on a subsequent admission were crossed over to the alternative study arm.
Statistical analysis
An episode of vaso-occlusive crisis was the unit of analysis of the study. Because of the mixed unpaired and paired observations as a result of the study-design, group differences were analyzed with mixed models (compound symmetry). In each model individual patients were subject variables and episodes of vaso-occlusive crisis were repeated variables. Dependent variables were the primary outcomes of the study with way of morphine administration (PCA or CI) as main factor. We further analyzed these differences using area under the curve (AUC) to adjust for duration of experienced side-effects. To that end, AUC variables were square root transformed so that normal distributions were obtained.
Change from baseline in QoL was a secondary outcome and was calculated by subtracting two days-outcomes in QoL from baseline-outcomes. All physiological and haematological parameters in this study are presented as medians and interquartile ranges (IQR). Since previous studies have demonstrated conflicting results on the effect of PCA on morphine consumption, the sample size of the study was calculated to detect a clinical relevant reduction of 50% in the mean hourly morphine consumption between the PCA-group and the CI-group. With a power of 0.90, we had to include at least 12 episodes of vaso-occlusive crisis in each study arm. Data were double entered into the study database and analyzed in SPSS version 11.5.1 for Windows.
Results

Patients
Twenty-five episodes of vaso-occlusive crisis in 19 patients were included in the study. Two patients (one randomized to CI and one to PCA) withdrew consent and requested treatment with meperidine after randomization (Figure 1). The base-line characteristics (Table 1) between the two groups were comparable except for the leukocyte count (15.2 (11.7-17.8) versus 11.3 (7.9-13.4) in the CI- and PCA-group, respectively). Homozygous SCD was the most common genotype in both groups (8/13 and 8/12 in the CI- and PCA-group, respectively). Median percentage of fetal haemoglobin, a major prognostic factor in SCD, was 3.7% in the CI-group and 3.2% in the PCA-group. Four episodes of vaso-occlusive crisis developed in patients taking hydroxyurea (two episodes in both groups). Of the six patients who crossed over, four received PCA and two received CI as initial treatment. The mean time between the first and second inclusion was five months.

Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>CI morphine</th>
<th>PCA morphine</th>
</tr>
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<tbody>
<tr>
<td>N</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>Age (y)</td>
<td>25 (20-33)</td>
<td>27 (23-42)</td>
</tr>
<tr>
<td>No. Female*</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Hydroxyurea treatment*</td>
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<td>2</td>
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<td>Haemoglobin genotype</td>
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</tr>
<tr>
<td>SS*</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>SC*</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Sβ*</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Baseline blood parameters</td>
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<td></td>
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<tr>
<td>Haemoglobin (mmol/L)</td>
<td>5.8 (5.3-6.9)</td>
<td>5.5 (4.4-7.0)</td>
</tr>
<tr>
<td>Leukocyte count (×10^9/L)</td>
<td>15.2 (11.7-17.8)</td>
<td>11.3 (7.9-13.4)</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>73 (55-79)</td>
<td>61 (50-78)</td>
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<td>C-reactive protein (mg/L)</td>
<td>12.7 (3.2-17.5)</td>
<td>9.0 (1.6-20.5)</td>
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<td>HbF (%)</td>
<td>3.7 (2.1-8.1)</td>
<td>3.2 (2.2-4.9)</td>
</tr>
<tr>
<td>HbS (%)</td>
<td>72 (47-85)</td>
<td>78 (62-90)</td>
</tr>
</tbody>
</table>

Data are presented as medians with interquartile ranges. *Number of patients.
Morphine dose and duration of treatment
The morphine dose in the PCA-group was significantly lower than the CI-group. The mean daily morphine consumption during treatment was 0.5(0.3-0.6)mg/hr in the PCA-group versus 2.4(1.4-4.2)mg/hr in the CI-group (P<0.001; figure 2) and median cumulative dose of morphine during vaso-occlusive crisis was lower in the PCA-group (33 (10-68)mg) than in the CI-group (260(204-529)mg; P=0.018; Table 2). This was partly explained by a relevant, but not statistically significant, reduced duration of morphine administration in the PCA-group compared to the CI-group (4.5(3.3-6.0) days versus 7.0(5.0-8.5) days; P=0.21) which was directly correlated to the total morphine dosage (P< 0.001). Assessment of the mean daily morphine consumption during the first three days of admission resulted in a median morphine dose of 0.5(0.3-0.8) mg/h in the PCA-group versus 3.5(2.0-4.5) mg/h in the CI-group (P<0.001). The patients in the PCA-group pressed the button to self-administer a dose of morphine on average 14 (9-16) times a day. Six of the patients in the PCA-group and five in the CI-group needed a dose increase because of inadequate pain relief. The median duration of admission in the PCA-group was 6.0(4.3-9.3) days and in the CI-group 9.0(6.0-12.0) days (P=0.15). Assessment of the morphine consumption if consecutive patients were unit of analysis in stead of an episode of vaso-occlusive crisis resulted in similar results (mean morphine consumption of 0.5(0.3-0.8) mg/h in the PCA group versus 2.5(1.5-4.7) mg/hr in the CI-group; P<0.001). The usage of additional oral pain medication was evenly distributed between the groups. In the CI-group three patients received tramadol and in the PCA-group two patients received tramadol.

| Table 2. Outcome on morphine consumption, pain score and quality of life. |
|-------------------------------------------------|-------------------------------------------------|---------------------|
| Treatment group                                   | CI morphine                                     | PCA morphine        |
| Morphine consumption and pain                     | 2.4 (1.4-4.2)                                   | 0.5 (0.3-0.6)       | 0.001   |
| Morphine dosage (mg/h)                            | 260 (204-529)                                   | 33 (10-68)          | 0.018   |
| Total morphine dosage (mg)                        | 4.9 (3.9-5.8)                                   | 5.3 (4.5-6.9)       | 0.09    |
| Mean verbal response pain score                   |                                                  |                     |         |
| Mean side-effect score and pain (AUC)*           |                                                  |                     |         |
| Nausea                                           | 18 (3-55)                                       | 11 (3-21)           | 0.045   |
| Constipation                                      | 45 (36-59)                                      | 30 (10-40)          | 0.021   |
| Pruritus                                          | 14 (0-28)                                       | 5 (0-25)            | 0.42    |
| Sedation                                         | 12 (6-33)                                       | 18 (0-20)           | 0.52    |

Data are presented as medians with interquartile ranges.* = Symptoms of side-effects are presented as area under the curve (AUC) during treatment.
Pain and Quality of Life

The range of pain scores were comparable in the two arms of the study (Figure 2B). In the CI-group the least verbal response pain score was 4.2(3.1-5.1), the mean score 4.9(3.9-5.8) and the worst score 5.8(4.5-6.2). These scores did not differ significantly from the least, mean and worst scores in the PCA-group, 4.2(3.4-5.8), 5.3(4.5-6.9) and 6.3(5.5-7.8) respectively. (P=0.14 P=0.09 and P=0.39). The mean pain score if patients were unit of analysis was 5.2(4.3-5.8) in the PCA-group versus 4.9(3.9-6.9) in the CI-group (P=0.85). Baseline pain-scores were comparable in both groups, 59(51-85) in the CI-group and 72(63-84) in the PCA-group and change from baseline after two days of treatment were also comparable respectively -24(-57 to -11) and -38(-52 to 4).

Assessment in quality of life remained unchanged in both groups (Table 3). There was a statistically significant difference in change over time in perceived importance of pain control between the PCA-group and the CI-group. Perceived importance of pain control increased in the PCA-group, whereas it decreased in the CI-group.

<table>
<thead>
<tr>
<th>Table 3. Individual response in pain score and quality of life after two days.</th>
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</thead>
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<tr>
<td></td>
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<td>Baseline</td>
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<td>Pain score (VAS)</td>
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<td>CI</td>
</tr>
<tr>
<td>PCA</td>
</tr>
<tr>
<td>Importance of pain control (VAS)</td>
</tr>
<tr>
<td>CI</td>
</tr>
<tr>
<td>PCA</td>
</tr>
<tr>
<td>Perceived pain control (VAS)</td>
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<td>Physical Health Summary</td>
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<td>CI</td>
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<tr>
<td>Mental Health Summary</td>
</tr>
<tr>
<td>CI</td>
</tr>
<tr>
<td>Quality of Life*</td>
</tr>
<tr>
<td>All numbers are medians (inter quartile range), *= Short Form Health Survey (SF36) **= P-value of difference in change after two days between continuous infusion (CI) and patient controlled analgesia (PCA) of morphine.</td>
</tr>
</tbody>
</table>

Side-effects and adverse events

The AUC of experienced nausea and constipation side-effect scores were significant lower in the PCA-group compared to the CI-group (respectively 11(3-21) versus 18(3-55) (P=0.045) and 30(10-40) versus 45(36-59) (P=0.021) (Table 2). No significantly difference in pruritus or sedation was found. After post hoc adjusting for morphine consumption no difference in side-effects were found between the two groups. Medians of mean oxygen saturation in the PCA-group and CI-group were respectively 98(95-99)% and 97(94-98)%. One patient in the CI-group experienced an episode of severe hypoxia due to hypoventilation and received an opioid antagonist (naloxone). Two intestinal pseudo-obstruction syndromes were reported in this study. Both occurred in the same patient (once in the PCA- and once in the CI-group). During eight episodes of vaso-occlusive
crisis acute chest syndrome was diagnosed. Five episodes were diagnosed in the CI-group (three before and two after randomization) and three episodes were diagnosed in the PCA-group (two before and one after randomization). None of these episodes required mechanical ventilation or resulted in hypoxia.

**Figure 2. Morphine dosage and range of painscores.**

(A): Mean morphine dosage (mg/h) during treatment in the CI-group was 2.4 (1.4-4.2) and in the PCA-group 0.5 (0.3-0.6) (P<0.001). (B): In the CI-group the least verbal response pain score was 4.2 (3.1-5.1) the mean score 4.9 (3.9-5.8) and the worst score 5.8 (4.5-6.2). These scores did not differ significantly from the least, median and worst scores in the PCA-group, 4.2 (3.4-5.8), 5.3 (4.5-6.9) and 6.3 (5.5-7.8) respectively (P=0.14, P=0.09 and P=0.39).
Discussion

Vaso-occlusive crisis in patients with SCD is characterized by intense and severe pain often requiring intravenous morphine. In this open randomized clinical trial, morphine administration with PCA resulted in markedly and significantly lower morphine consumption as compared with CI with a similar effect on pain or other scales of relief. Patients in the CI-group needed about 5 times more morphine per hour as compared to the PCA-group. Additionally, the difference in total cumulative morphine consumption was even larger since the duration of treatment tended to be shorter in the PCA-group. Total experienced nausea and constipation depicted in the AUC of the corresponding side-effects scores during treatment were significant lower in the PCA-group compared to the CI-group. This difference disappeared after correcting for morphine consumption confirming that these side-effects are related to the cumulative morphine consumption. No differences in pruritus and sedation were observed between the groups. The results of this trial are in accordance with the observation of Gonzalez et al. demonstrating that PCA was effective and safe in patients with SCD with vaso-occlusive crisis in a day care setting as compared to intermittent morphine injections for maximal 8 hours, although no difference in the total amount of morphine was demonstrated in that study.7 Besides this study, no other comparative trials have been performed with PCA in patients with SCD. A retrospective chart review in 26 children with SCD and vaso-occlusive crisis demonstrated that children treated with a PCA-regimen with low basal rate infusion and high bolus dose used significantly less morphine during their hospitalization, had a shorter length of hospital stay, and reported lower pain scores as compared to a PCA regimen with a high basal rate infusion and a low bolus dose.8 A possible explanation for this effect, which also could explain the low morphine consumption in the PCA-treated patients in the present study, may be that a more rapid analgesic effect is induced by bolus administration of morphine rather than CI. However, comparative trials with PCA in patients with other causes of pain have demonstrated conflicting results on the cumulative morphine consumption. 9,20 We also analyzed quality of life during treatment to assess response on other outcomes than verbal response pain scale. No difference in response in quality of life was observed between the intervention groups. A statistically significant difference was demonstrated in perceived importance of pain control. Patients assigned to PCA showed a slight increase in perceived importance of pain control while patients assigned to CI showed a decrease. This may indicate that patients treated with PCA experience the direct control of morphine administration to be more important than patients in the control group, despite similar pain intensity scores in the two groups. An alternative explanation for this finding may be “post decision justification bias”.21 This is a cognitive adaptation mechanism by which patients justify the way they were managed. Since pain control is a key-characteristic of PCA, patients who were managed with PCA are expected to value pain control.
as more important than patients who were managed with CI. Psychological factors which influence response to treatment in the context of illness and pain are complex. Control may have a positive influence on these factors.\textsuperscript{22} This factor may be particularly important for patients with SCD and may be a possible explanation for the fact no difference in pain or quality of life was found between the CI-group and PCA-group despite the large difference in the amount of morphine used.\textsuperscript{23,24} Furthermore, patients in the PCA-group appeared to accept a pain score of 5.5 and did not titrate to achieve a pain free state, while most health professionals titrate pain medication to reduce even mild pain. This was observed in previous studies in children as well as adults.\textsuperscript{25-27} The analgesia-induced side effects as perceived by the patient may be an important reason for this phenomenon. Hypothetically, patients balance their pain with the side-effects of morphine administration.\textsuperscript{28} In the study, we found that the higher consumption of morphine in the CI group resulted in significant more morphine-related side-effects like nausea and constipation. Although no significant difference in sedation and pruritis was demonstrated it is likely that also these side-effects would be significantly less if a larger samples size would have been studied.

Some aspects of our study require comment. Firstly, the limited sample size of our study improves the chance of unevenly distributed confounders. However, after post-hoc correcting for possible confounders as age, gender, leukocyte count or genotype the difference in morphine usage stays clinical and statistical significant. Furthermore, the study design of mixed paired and unpaired observations decreases unevenly distribution of unknown confounders. Although our study is of comparable size as other trials with PCA a larger trial will be needed to demonstrate whether the use of PCA in vaso-occlusive crisis will result in a reduction in length of hospital stay and complications such as acute chest syndrome.\textsuperscript{9-20} Secondly, the patient-controlled-analgesia design of our study made a complete blinding of the study impossible and could therefore be subject to bias. Thirdly, we took episodes of vaso-occlusive crisis as unit of analysis. To prevent multiple enrolments of a few patients with frequent vaso-occlusive crises, patients were only allowed to participate in the study twice and were treated according to the opposite arm of the study upon second admission. However, if patients were the unit of analysis, our study resulted in similar conclusions.

In conclusion, the present study demonstrates that the use of PCA in patients with SCD with vaso-occlusive crisis results in a significant reduction in morphine consumption with equivalent response on measurements of pain and quality of life. A significant difference in morphine-induced side-effects was found and PCA may therefore be considered the first choice for adequate morphine administration in patients with SCD with vaso-occlusive crisis.
Reference List


Owen H, Kluger MT, Plummer JL. Variables of patient-controlled analgesia: the relevance of bolus dose size to supplement a background infusion. Anaesthesia 1990; 45(8):619-622.


Sickle cell disease related organ damage occurs irrespective of pain rate; Implications for clinical practice

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on behalf of the CURAMA study group.

¹Department of Hematology, Academic Medical Center, Amsterdam.
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⁴Department of Hematology, Erasmus Medical Center, Rotterdam.
Abstract

In daily clinical practice, the frequency of painful crises (pain rate) is considered an important parameter of sickle cell disease (SCD) severity. We assessed the prevalence of SCD-related organ damage and complications and their relation to pain rate. Organ damage and the history of vaso-occlusive complications were obtained via systematic screening of consecutive patients and by chart review. In 104 adult sickle cell patients pain rate was related to a history of acute chest syndromes, avascular osteonecrosis, iron overload, priapism and cholelithiasis. However, major disease related complications such as microalbuminuria and pulmonary hypertension were detected in 23% and 24%, respectively, of patients without painful crises in the study period and were not related to pain rate. The occurrence of several major SCD related disease manifestations is not related to painful crisis frequency underscoring the importance of systematic screening for developing organ damage in sickle cell patients irrespective of pain rate.
Introduction

Sickle cell disease (SCD) is heterogeneous in its presentation, with differences in the rate and severity of complications not only between different genotypes but within a single genotype as well. Even patients with the most severe genotype, HbSS, may vary in their clinical presentation from being continuously admitted for the management of acute complications to rarely requiring medical care. Both vaso-occlusion and chronic haemolysis are major determinants of SCD related organ damage.1 With the increasing life expectancy of sickle cell patients in the Western world the effect of accumulating organ damage on the quality of life and life expectancy is becoming an important factor in managing SCD.2 Early recognition of developing organ damage is imperative in order to institute specific therapeutics in a timely manner. However, a landmark autopsy study in sickle cell patients demonstrated a high prevalence of organ damage that often not recognized during life by treating physicians.3 Although the frequency of the painful sickle cell crisis, which is the hallmark SCD related clinical complication, is considered a parameter of disease severity, most patients do not frequently experience painful crises that require medical care. Nonetheless, in general sickle cell patients have a significantly reduced life expectancy suggesting that clinically significant organ damage accumulates irrespective of the pain rate.4 As the pain rate is considered an important parameter of SCD severity we analyzed whether the prevalence of SCD-related manifestations is related to the frequency of painful crises.
Design and Methods

Patients
Adult sickle cell patients visiting the Department of Haematology of the Academic Medical Center (AMC) in Amsterdam were considered eligible. After obtaining written and informed consent, patients were screened for SCD-related manifestations from July 2005 until December 2006 as defined below. This study was approved by the internal review board of the AMC and carried out in accordance with the principles of the Declaration of Helsinki.

SCD related manifestations
SCD-related manifestations were assessed by systematic screening and medical record review and defined as follows:
Microalbuminuria: urinary creatinine (mmol/l) to urinary albumin(mg/l) ratio >3.5 (males)/ >2.5 (females) confirmed with 24 hour urine collection with microalbuminuria >30 mg/24 hours. Renal failure: creatinine clearance <100 ml/min (Cockcroft and Gault). Pulmonary hypertension (PHT): tricuspid regurgitation jet flow velocity (TRV) ≥2.5 m/s in rest detected by Doppler echocardiography. PHT was considered absent with no or only trace TRV. Retinopathy: presence of at least mild non-proliferative retinopathy. Perceptive hearing loss: loss of >20 dB with no other explanation than SCD. Cholelithiasis: presence of gallstones (ultrasound) or previous cholecystectomy because of cholecystolithiasis. Iron overload: plasma ferritin level >1000 μmol/L (on at least three occasions during steady state) and a history of >20 transfused packed cells. Acute chest syndrome (ACS): defined as previously described occurring between January 2002-January 2007. Symptomatic avascular osteonecrosis: local pain and reduced function documented osteonecrosis of the femoral or humeral head (hip or shoulder X-ray) or a history of surgical intervention for osteonecrosis. Leg ulcers: chronic ulcers of the ankle not otherwise explained. Priapism: spontaneous painful erection requiring hospital care. Stroke: history of stroke confirmed by Magnetic Resonance Imaging or Computerized Tomography.

Pain rate
Pain rate was assessed by calculating the cumulative number of admissions for painful crises (defined as typical musculo-skeletal/abdominal pain not otherwise explained) from January 2002 until January 2007 and categorizing patients into three groups: no crises, less than one crisis, or one or more crises a year (on average). Painful crises not requiring medical care were excluded.
Laboratory parameters
All laboratory data were obtained during routine outpatient visits at least 4 weeks after the last acute disease related complication or blood transfusion. Foetal haemoglobin percentage (HbF%) was determined by cation-exchange high performance liquid chromatography\textsuperscript{13}, and $\alpha$-thalassaemia screening was performed with a multiplex PCR assay.\textsuperscript{14}

Statistical analysis
The most severe SCD genotypes (HbSS and HbS$\beta^0$-thalassaemia) were grouped together, as were the relatively genotypes (HbSC and HbS$\beta^+$-thalassaemia). Continuous data are presented as medians with their corresponding interquartile range (IQR). Between group differences were tested with the Mann-Whitney U test. Categorical data are presented as percentages with between group differences or statistical dependence tested with Fishers’ Exact Test. Bivariate correlations of ordinal data were tested by determining the Spearman correlation coefficient ($r_s$). P-values below 0.05 were considered statistically significant. SPSS 12.0.2 (SPSS Inc, Chicago, IL) was employed.
Results and Discussion

One hundred and ten adult sickle cell patients were eligible of whom 6 were excluded due to incomplete data collection, leaving 104 included sickle cell patients (see Table 1).

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<thead>
<tr>
<th>Table 1. Patient characteristics</th>
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<th>HbSC (n=29)/$S^+$/thal (n=11)</th>
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<tbody>
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<td>N</td>
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<td>Age (year)</td>
<td>27 (21-41)</td>
<td>29 (24-38)</td>
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<td>Haemoglobin (g/dL)</td>
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<td>11.3 (10.6-12.2)</td>
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<td>Reticulocytes (%)</td>
<td>8.2 (5.9-10.9)</td>
<td>2.8 (2.2-3.8)</td>
<td>&lt;0.001</td>
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<td>Leukocytes (×10 9/L)</td>
<td>9.0 (7.2-11.7)</td>
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<td>Fetal Haemoglobin (%)</td>
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<td>Lactate Dehydrogenase (U/L)</td>
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<td>Creatinine (umol/L)</td>
<td>51 (42-57)</td>
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<td>Organ damage (%)</td>
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<td>47</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>- one or more</td>
<td>27</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Stroke</td>
<td>11</td>
<td>0</td>
<td>0.042</td>
</tr>
<tr>
<td>Priapism (% of males)</td>
<td>21</td>
<td>6</td>
<td>0.206</td>
</tr>
</tbody>
</table>

Results are medians (interquartile range). *Mann-Whitney-test or Two-sided Fisher-exact test.
Apart from retinopathy (which was more prevalent in HbSC/HbSβ⁺-thalassaemia patients), most manifestations of SCD were significantly more often present in HbSS/HbSβ₀-thalassaemia patients. PHT was detected in 32% and 12% of the HbSS-HbSβ₀ and HbSC/HbSβ⁺ respectively, with a median TRV of 2.60 (2.50-2.69) m/s. None of these patients had severe PHT (TRV>3.0 m/s). Although significantly more patients with frequent sickle cell crises used hydroxyurea, no difference in SCD-related organ damage was observed between patients with or without hydroxyurea. (data not shown).

Avascular osteonecrosis, a history of ACS, priapism and cholelithiasis, as well as iron overload were significantly related to pain rate (table 2). The association between iron overload and the pain rate is likely the result of liberal blood transfusions for treating painful crises prior to instituting evidence based management protocols for SCD in the Netherlands.¹⁵ Importantly, microalbuminuria and PHT were detected in 23% and 24% respectively of patients without painful crises during the study period. Furthermore, PHT and microalbuminuria were detected in 23% and 10% respectively of patients with no painful crises in the last five years. These patients did not have leg ulcers, episodes of priapism or ACS in the last 5 years and appeared clinically well based upon history taking and physical examination. Such patients would likely have been misclassified as having mild SCD. These data indicate that several major disease related complications⁶,¹⁰,³⁰,³¹ are not related to pain rate and occur even in a significant number of patients that seem clinically well, underscoring the importance of systematic screening for SCD-related complications even in clinically mildly affected patients.

Several shortcomings of this study need to be addressed. Firstly, the history of acute painful crises was limited to the last 5 years and only painful crises for which patients were admitted were evaluated. Therefore the conclusions may not be extrapolated for the number of painful crises experienced at home or before the evaluated 5 year period. Secondly, selection bias has likely occurred given the retrospective nature of this study. Thirdly, since this study was performed in a tertiary teaching hospital referral bias cannot be excluded. However, given the similar prevalence of most SCD-related disease manifestations in our cohort to that reported in literature⁴,⁸,¹⁶-²³ it seems representative. The prevalence of renal failure may, however, be underestimated as the characteristic supranormal proximal tubular function characteristic of SCD likely results in an overestimation of glomerular filtration.²⁴ Also, the prevalence of retinopathy in our study is higher as compared to previous reports which is likely due to the inclusion of mild non-proliferative retinopathy.²⁵ Lastly, other forms of sickle cell related organ damage such as pulmonary, hepatic and neurocognitive organ damage have not been analysed in this study. Nonetheless, we feel that the aforementioned factors do not influence the main findings of our study.

In conclusion, clinically relevant forms of organ damage such as PHT and micro-albuminuria, occur irrespective of the frequency of painful crises in adults with SCD. Systematic screening for and evaluation of organ damage in all sickle cell patients seems indicated since many of the sickle
cell-related complications may otherwise go unnoticed, thereby delaying the institution of potential therapeutic measures.

Table 2. Prevalence of sickle cell related complications.

<table>
<thead>
<tr>
<th></th>
<th>No. of crises/year</th>
<th></th>
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<th>P*</th>
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<tr>
<td></td>
<td>0</td>
<td>0-1</td>
<td>≥1</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>32</td>
<td>47</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Alpha-thalassaemia (%)</td>
<td>27</td>
<td>37</td>
<td>50</td>
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<tr>
<td>Sex (% male)</td>
<td>34</td>
<td>43</td>
<td>36</td>
<td>0.851</td>
</tr>
<tr>
<td>Hydroxyurea use (%)</td>
<td>13</td>
<td>11</td>
<td>36</td>
<td>0.014</td>
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<tr>
<td>Organ damage (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Microalbuminuria</td>
<td>23</td>
<td>21</td>
<td>25</td>
<td>0.906</td>
</tr>
<tr>
<td>Renal failure</td>
<td>3</td>
<td>9</td>
<td>4</td>
<td>0.819</td>
</tr>
<tr>
<td>Pulmonary hypertension</td>
<td>24</td>
<td>32</td>
<td>16</td>
<td>0.614</td>
</tr>
<tr>
<td>Retinopathy</td>
<td>43</td>
<td>39</td>
<td>35</td>
<td>0.602</td>
</tr>
<tr>
<td>Perceptive hearing loss</td>
<td>16</td>
<td>17</td>
<td>6</td>
<td>0.376</td>
</tr>
<tr>
<td>Iron overload</td>
<td>6</td>
<td>6</td>
<td>24</td>
<td>0.041</td>
</tr>
<tr>
<td>Cholelithiasis</td>
<td>32</td>
<td>48</td>
<td>75</td>
<td>0.002</td>
</tr>
<tr>
<td>Clinical complications (%)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Avascular osteonecrosis</td>
<td>3</td>
<td>13</td>
<td>24</td>
<td>0.019</td>
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<tr>
<td>Leg ulcers</td>
<td>13</td>
<td>6</td>
<td>8</td>
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<tr>
<td>Acute chest syndrome</td>
<td>0</td>
<td>0</td>
<td>40</td>
<td>&lt;0.001</td>
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<tr>
<td>Stroke</td>
<td>9</td>
<td>2</td>
<td>12</td>
<td>0.803</td>
</tr>
<tr>
<td>Priapism (% of males)</td>
<td>9</td>
<td>5</td>
<td>44</td>
<td>0.041</td>
</tr>
<tr>
<td>Genotype (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HbSS/S$^s$-thal</td>
<td>53</td>
<td>64</td>
<td>68</td>
<td>0.241</td>
</tr>
<tr>
<td>HbSC/S$^s$-thal</td>
<td>47</td>
<td>36</td>
<td>32</td>
<td>0.241</td>
</tr>
</tbody>
</table>

Numbers are percentages. * P-value based on Spearman rank test.
Sickle cell disease related organ damage occurs irrespective of pain rate

Reference List


Sickle cell disease related organ damage occurs irrespective of pain rate
Normal sublingual microcirculation during painful crisis in sickle cell disease
(Based on: Microvasc Res. 2008 Mar 12. [Epub ahead of print])

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Abstract

Obstruction of the microcirculation is the most important cause of painful crisis in sickle cell disease (SCD). Extensive microvascular obstruction has been observed in mouse models of SCD. A technique to determine the extent of the microcirculatory obstructions in humans may be helpful in the clinical setting and for research purposes. Therefore, we measured sublingual microcirculation longitudinally in patients with SCD admitted with painful crisis. Sublingual microcirculation was recorded with side-stream darkfield (SDF) imaging and semi-quantified with a microvascular flow index (MFI) on a range from 0 to 4 (arbitrary units; from 0 (no flow) to 4 (hyperdynamic flow)).

Thirteen consecutive adult sickle cell patients admitted with painful crises were included and provided 47 measurements of MFI in 14 episodes of painful crisis. Seven patients provided baseline measurements and seven healthy controls were studied. The mean (± standard error of the mean) MFI during painful crisis was 2.6±0.1 and did not change during the painful crisis. The mean MFI of patients with SCD during steady state (2.7±0.1) and the mean MFI of the controls (2.7±0.1) were not different from the mean MFI during painful crisis. During painful crisis irregular microvascular perfusion, expressed by the distribution width of the microvascular blood flow velocity, correlated negatively (r=-0.484; P=0.002) with hemoglobin concentration.

We conclude that sublingual microcirculatory blood flow velocity is not disturbed in sickle cell patients during painful crisis.
Introduction

Vaso-occlusive complications account for a major part of the morbidity suffered by patients with sickle cell disease (SCD) and has been demonstrated to be a major determinant of life expectancy. The most frequent vaso-occlusive complication is the painful crisis. In painful crises, widespread microcirculatory obstruction leads to ischemic bone pain, resulting in frequent hospital admissions for supportive care. Since objective tools to quantify the extent of the microcirculatory obstruction during the painful crises are not available, frequency and duration of painful crises is often used to assess the severity of crises and effect of therapy. A technique to determine the extent of the microcirculatory obstruction during crisis may therefore be very helpful in both clinical setting and for research purposes.

Despite the fact that the pain in vaso-occlusive crisis is mainly located in bones, animal models of SCD, which are frequently used to investigate the pathophysiology of SCD and painful crisis, have demonstrated that microcirculatory disturbances are not limited to the microvasculature of the bones.\(^{1-5}\) Previously, microcirculatory obstruction during painful crisis has been visualized in sickle cell patients at the vascular beds of the nailfold and conjunctiva but resulted in conflicting conclusions.\(^{6-8}\)

The Orthogonal Polarized Spectral imaging technique (OPS) is a new technique to visualize the microcirculatory blood flow velocity with better optical density on easy accessible mucosal membranes such as the sublingual tissue. This technique has been successfully used to quantify sublingual microcirculatory disturbances in different diseases.\(^{9-14}\)

In the present study, we explored the sublingual microcirculatory blood flow velocity in SCD during steady state and painful crisis using Side-stream Dark Field (SDF) imaging, an improved version of OPS.\(^{15-17}\)
Methods and materials

Patients
Consecutive sickle cell patients aged 17 years or older (HbSS, HbSC, HbSβ0-thalassemia and HbSβ+ thalassemia) admitted with a painful crisis to the Academic Medical Center in Amsterdam, the Netherlands were included. A painful crisis was defined as the presence of typical bone pain which could not be explained otherwise. Sickle cell crises were divided in three artificial periods of admission defined as period I (day 1-3), period II (day 4-6) and period III (day 7-10). Patients with oral mucosal infections or trauma were excluded. Patients served as their own controls by assessment of the sublingual blood flow velocity at steady state, at least 4 weeks following the painful crisis. In addition, healthy individuals without SCD served as controls. The study was approved by the local medical ethics committee and all participating patients gave written informed consent. The study was carried out in accordance with the principles of the Declaration of Helsinki.

Measurement of sublingual microcirculation
The sublingual microcirculation was analyzed using SDF imaging (Microscan, MicroVision, Amsterdam, The Netherlands) as described by others. In short, the SDF-probe, containing the camera and light source, is put gently under the middle of the tongue. SDF uses light emitting diodes (LED’s) emitting (green) light with a wavelength of 530 nm, which is completely absorbed by hemoglobin causing erythrocytes to appear as dark particles. The sublingual microcirculation was measured daily from admission until a maximum of 10 days thereafter. Intravital microscopy images were recorded using a digital video recorder (Sony SDR-20 P). The images had to be sufficiently sharp and the probe should not have exerted any pressure on the tissue examined. From each of these video recordings fragments were selected by a second investigator unaware of the clinical condition of the patient or control. These fragments had to consist at least 5 seconds of steady, clear and sharp images in order to measure Microvascular flow index (MFI) (see below). The fragments were edited using Microsoft® Windows ® Movie Maker® (version 5.1, service pack 2).

Microvascular flow index (MFI)
The microvascular flow index (MFI) is a previously described semi-quantitative analysis method to determine the blood flow velocity through the microvasculature of specific organs. Video fragments recorded using SDF were divided into quadrants (figure 1A). In each of the quadrants blood vessels were subdivided into the diameter categories small (6-15 μm), medium (15-25 μm) and large (25-50 μm) (figure 1B). For analysis of the microcirculation only the diameter categories
small and medium were studied. Flow velocity of erythrocytes in each individual blood vessel was estimated by trained observers and categorized as follows: 0: no flow, 1: intermittent flow, 2: stuttering flow, 3: normal flow and 4: hyperdynamic flow. To determine the average flow in both categories of blood vessels (small and medium sized blood vessels) the MFI was calculated in all quadrants per video fragment. Per measurement at least three video fragments were analyzed and averaged. All fragments were scored by the same observer. Since hyperdynamic flow in some vessels may compensate for limited or obstructed flow in other vessels resulting in a similar mean MFI, also the distribution width of blood flow velocity, defined as the standard deviation (SD) of the measured MFI, and the total number of occluded (0) and vessels with intermittent blood flow velocity (1) per fragment were assessed.

![Image](image.png)

**Figure 1. Analysis video images.**
Captured video images are divided in four quadrants (I, II, III and IV). Classification of blood vessels by size; Large (L3; >25μm), medium (M2; 15-25μm), small (S3; 6-15μm) blood vessels.

**Inter and intra observer variability**
Analysis of the SDF video fragments was tested for inter observer variability by randomly letting an experienced second observer analyzed a subset of SDF video fragments in a blinded fashion. Both observers were unaware of the other observers MFI estimations. Intra observer variability was tested by re-analyzing a random set of SDF video fragments three weeks after the first analysis by the same observer.
Statistics

The average daily MFI and flow distribution during hospital admissions for painful crisis were compared with base-line measurements and controls using student’s T-test and ANOVA. The inter and intra observer variability was determined by calculating the correlation coefficient as described by Bland and Altman.\textsuperscript{19} All data are presented as means with standard errors and were statistically analyzed using SPSS 12.0.02 for Windows\textregistered (released 24 March 2004).
Results

Patients
Thirteen adult sickle cell patients (7 HbSS, 2 HbSC, 3 HbSβ+-thalassemia 1 HbSβ0-thalassemia, mean age 37±13 years) provided 47 measurements of microcirculatory flow during 14 episodes of painful crises. Seven patients were reassessed during steady state condition to provide baseline measurements. Seven healthy controls were also included.

![Diagram showing MFI values]

Figures 2 & 3. Flow measurement between groups and during painful crisis.
Figure 2: Mean Microvascular Flow Index (MFI) in patients with SCD during painful crisis (Crisis) and during steady state (Steady state) compared to healthy controls (Controls). No difference in MFI was demonstrated between the different groups. Figure 3: Mean Microvascular Flow Index (MFI) in patients with SCD during painful crisis. Day 1-3 represents the first 3 days after admission for painful crises, day 4-6 and day 7+ represent the days following. No difference in MFI was demonstrated between the clinical periods of the painful crises.

Microvascular Flow Index (MFI)
The microvascular blood flow velocity expressed as the MFI appeared to be similar in sickle cell patients during steady state (2.6 ± 0.1) and painful crisis (2.7 ± 0.1) (figure 2). In addition, no differences could be detected between the MFI in sickle cell patients and healthy controls (2.7±0.1). During painful crises the MFI measured on subsequent days did not change.(figure 3) Also the flow pattern, as presented in figure 4, and the distribution width of the microvascular flow velocity did not differ between the groups, excluding the possibility that hyperdynamic flow in some vessels may have compensated for the limited or obstructed flow in other vessels. During painful crisis, the distribution width of the microvascular blood flow velocity was negatively
correlated with hemoglobin level (r=-0.484; P=0.002) and positively correlated with leukocyte count (r=0.438; P=0.014) and lactate dehydrogenase plasma levels (r=0.417; P=0.027) and also the total number of blood vessels with no or intermittent flow was negatively correlated to hemoglobin concentration (r=-0.367; P=0.028). (Figure 5). Using a multivariate analysis to correct for genotype, the above mentioned correlations remained significant except for the correlation between LDH and distribution width. No difference in distribution width of the microvascular blood flow velocity was found between patients and controls.

**Inter- en intra observer variability**

The calculated correlation coefficient for intra-observer variability of small and medium vessels were 0.965 (P< 0.001) and 0.967 (P<0.001) respectively (corresponding Bland and Altman’s limits of agreement -0.31 to 0.21 and -0.56 to 0.35). The calculated correlation coefficient for inter-observer variability of small and medium vessels were 0.836 (P = 0.001) and 0.810 (P<0.001) respectively (corresponding Bland and Altman’s limits of agreement -0.38 to 0.30 and −0.77 to 0.57).

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**Figure 4 & 5. Flow distribution pattern and correlations during painful crisis.**

Figure 4: The total number of microvascular blood vessel with either: no flow (0), stuttering flow (1), slow flow (2), normal flow (3) or hyperdynamic flow (4). “T” represents the mean of all flow patterns within the groups. Data are expressed as total number of blood vessels observed ± SD. No statistically significant difference was observed between the groups.

Figure 5: During painful crisis, the distribution width of the microvascular blood flow velocity was negatively correlated (Spearman rank test) with hemoglobin level (r=-0.484; P=0.002). *: The flow distribution width was defined as the standard deviation (SD) of the measured MFI.
Discussion

In this study, we measured sublingual microvascular blood flow velocity in patients with SCD during painful crisis and steady state with a new optical technique, called SDF imaging, to visualize the microcirculation. In addition, healthy controls were analyzed. No difference in sublingual microvascular blood flow velocity was observed between sickle cell patients during painful crisis and steady state or between sickle cell patients and healthy volunteers. Since also the distribution of blood flow velocity expressed by the MFI distribution width, was similar between all groups, our findings can not be explained by hyperdynamic blood flow in some microcapillaries compensating for stuttering flow in other microcapillaries. This confirms the observations of Lipowski et al. who did not find differences in blood flow velocity distribution between sickle cell patients in steady state, during crisis or healthy volunteers either. 

Our findings are in contrast with the previous observations. Cheung et al. demonstrated a reduction in the bulbar microvascular blood flow velocity during painful crisis while the opposite was observed by Lipowski et al. in the vascular bed of the nailfolds. We considered a number of explanations for this observation. Firstly, a new technique of intravital microscopy was used in our study. However, it seems unlikely that this technique explains the difference since the SDF technique used in this study provides better image quality than conventional OPS intravital capillary microscopy. Secondly, the quantification methods of the microvascular blood flow may not have been sensitive enough to detect a disturbed microvascular flow in sickle cell patients. However, in the study of Cheung et al. microvascular flow velocities appeared to decrease 46% during crisis while in our study, the quantification method was powered to detect a difference in MFI of at least 0.3 representing a 10% reduction in the microvascular blood flow velocity. In addition, no difference in flow distribution in the microcapillaries was observed between patients in steady state and during sickle cell crisis. Thirdly, our results may be explained by the fact that sublingual microvascular blood flow is not hampered in SCD. Sublingual microvascular blood flow is considered to be a reliable estimation of systemic microvascular blood flow in septic patients and has demonstrated to predict survival in patients with severe heart failure. Lastly, our study may have missed actual changes in microcirculatory blood flow due to the relative small sample size. Interestingly, the distribution width of the microvascular blood flow velocity during painful crises appeared to be inversely related to hemoglobin levels indicating a more irregular microvascular blood flow pattern in patients with severe anemia. The elevated distribution width of the microvascular blood flow velocity of subjects with severe anemia may represent a degree of overcompensation for the rheological insult in capillaries that are occluded during painful crisis. The flow distribution on average however did not differ between patients and controls. This may be caused by normal physiological changes in flow through different capillaries at normal to high hematocrit which has also been observed by Lipowsky et al. and Cheung et al.
We conclude that sublingual microcirculatory blood flow velocity is not disturbed in sickle cell patients during painful crisis per se and is therefore not useful to quantify the severity of painful crisis in sickle cell disease.
Reference List

Part II: Pathophysicsiology
Circulating erythrocyte-derived microparticles are associated with coagulation activation in sickle cell disease

(Submitted)

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Chapter 5

Abstract

Sickle cell disease (SCD) is characterized by a hypercoagulable state involving multiple factors, including chronic hemolysis and circulating cell-derived microparticles (MPs). There is still no consensus on the cellular origin of such MPs and the exact mechanism by which they may support coagulation activation in SCD.

In the present study, we analyzed the origin of circulating MPs and their pro-coagulant phenotype during painful crises and steady state in 25 consecutive SCD patients. The majority of MPs originated from platelets (GPIIIa, CD61+: 86.1%) and erythrocytes (Glycophorin A, CD235+: 9.7%), and their numbers did not differ significantly between crisis and steady state. Erythrocyte-MPs strongly correlated with plasma levels of hemolytic markers, i.e. hemoglobin (r=-0.58, P<0.001) and lactate dehydrogenase (r=0.59; P<0.001), von Willebrand factor as a marker of endothelial activation (r=0.44; P<0.001), and D-dimer and prothrombin fragment F1+2 (r=0.52; P<0.001 and r=0.59; P<0.001, respectively) as markers of fibrinolysis and coagulation activation. Thrombin generation depended on the total number of MPs (r=0.63; P<0.001). Anti-human factor XI inhibited thrombin generation by about 50% (P<0.001), whereas anti-human factor VII was ineffective (P>0.05). The extent of factor XI inhibition was associated with erythrocyte-derived MPs (r=0.50; P=0.023).

We conclude that the procoagulant state in SCD is partially explained by the factor XI-dependent procoagulant properties of circulating erythrocyte-derived MPs.
Introduction

Sickle cell disease (SCD) is characterized by chronic hemolysis and recurrent ischemia due to micro-vascular occlusion following the adhesion of erythrocytes and leukocytes to the vascular endothelium.\(^1\) In addition, SCD is complicated by chronic coagulation and endothelial activation, resulting in a hypercoagulable state.\(^2\) Although this hypercoagulability is considered to be multifactorial, it has become increasingly clear that chronic hemolysis plays a pivotal role in this process and many other sickle cell-related complications. Also in other diseases characterized by chronic hemolysis, like paroxysmal nocturnal hemoglobinuria (PNH) and β-thalassemia, hemolysis has been related to coagulation activation and thrombotic complications.\(^2\)^\(^-\)\(^3\) This was confirmed in a recent study demonstrating that the hypercoagulable state in SCD is specifically linked to the rate of phosphatidylserine (PS) exposure on erythrocytes.\(^4\)

Previous observations also suggested a possible contribution of circulating cell-derived microparticles (MPs) to the hypercoagulable state in SCD.\(^5\) MPs are small membrane vesicles released from cells by “budding” upon activation or during apoptosis, and in blood MPs are encountered originating from platelets, erythrocytes, leukocytes and endothelial cells.\(^6\) Elevated numbers of circulating MPs have been reported in patients suffering from a variety of diseases with vascular involvement and hypercoagulability including SCD.\(^7\)^\(^-\)\(^13\) The exact mechanism by which circulating MPs trigger coagulation in SCD, however, remains unclear. The majority of circulating MPs in SCD originates from erythrocytes and platelets and may support coagulation activation by exposure of phosphatidylserine (PS) to facilitate complex formation between coagulation factors in the coagulation activation cascade, while others demonstrated an increased exposure of tissue factor (TF) on monocyte-derived MPs.\(^7\)^\(^-\)\(^14\) A more thorough understanding of the mechanism by which circulating MPs affect coagulation and endothelial activation might be helpful in the development of new therapeutic therapies in SCD.

In the present study, we established the cellular origin of circulating MPs in patients with SCD during painful crises and in the chronic phase, and explored their relation with coagulation, fibrinolysis and endothelial activation.
Methods

Patients
Consecutive adult sickle cell patients (HbSS, HbSβ0/+-thalassemia or HbSC, confirmed with high performance liquid chromatography), admitted with a painful crisis in the Academic Medical Center (AMC) in Amsterdam were eligible for inclusion. A painful crisis was defined as hospital admission for the treatment of pain in the extremities, back, abdomen, chest, or head not otherwise explained. Patients were asked to provide a blood sample every second day during admission to explore patterns in number and origin of MPs during painful crises. Patients included during a painful crisis were asked to provide a baseline blood sample during a subsequent visit to the outpatient clinic. Baseline (steady state) was defined as a period without pain or painful crisis for at least four weeks. All patients gave written informed consent and this study was approved by the internal review board of the AMC. The study was carried out in accordance with the principles of the Declaration of Helsinki.

Collection of blood samples
Blood samples were taken from the antecubital vein without tourniquet through a 19-gauge needle with a vacutainer system. Blood was collected into a 4.5 mL tube containing 0.105M buffered sodium citrate (Becton Dickinson, San Jose, CA). Within 15 minutes after collection, cells were removed by centrifugation for 20 minutes at 1550 x g at 20°C. Plasma samples were then divided into 0.25 mL aliquots, immediately snap frozen in liquid nitrogen and stored at -80°C.

Reagents and assays
Fluorescein isothiocyanate (FITC)-labelled IgG1, phycoerythrin (PE)-labelled IgG1, CD20-PE, CD14-PE and CD71-PE were obtained from Becton Dickinson (San Jose, CA), IgG2b-PE from Immuno Quality Products (Groningen, The Netherlands), CD61- FITC from Pharminingen (San Jose, CA), CD54-PE and CD62P-PE from Beckman Coulter Inc. (Fullerton, CA), CD62E-PE from Ancell Corporation (Bayport, MN), CD106-FITC from Calbiochem (Gibbstown, NJ), CD142-FITC from American Diagnostica Inc. (Stamford, CT), CD144-FITC from Alexis Biochemicals (San Diego, CA) and (anti-)glycoporphin A (CD235) from DAKO (Glostrup, Denmark). Finally, allophycocyanin (APC)-conjugated annexin V was purchased from Caltag (Burlingame, CA). Anti-factor VII, anti-factor XI and anti-TFPI were obtained from Sanquin (Amsterdam, The Netherlands). Assays were performed as described by the manufacturer (Parameter human sP-Selectin Immunoassay by R&D Systems; Minneapolis, MN, USA). Platelet counts were determined with a Cell-Dyn 4000 (Abbott Diagnostics Division; Abbott Laboratories; Hoofddorp, The Netherlands). Markers of coagulation activation, fibrinolysis and endothelial
activation (prothrombin fragment F$_{1+2}$ (F$_{1+2}$) Enzygnost, Dade Behring, Marburg, Germany; von Willebrand Factor (VWF-ag) antibodies from DAKO, Glostrup, Denmark; D-dimer, Asserachrom D-Di, Roche, Almere, the Netherlands) were measured by ELISA.

**Isolation of microparticles**

A sample of 250 μL frozen plasma was thawed on melting ice for one hour and centrifuged for 30 minutes at 18,890x g and 20°C to pellet the MP. After centrifugation, 225 μL of the supernatant was removed. The pellet and remaining supernatant were resuspended in 225 μL phosphate-buffered saline containing citrate (154 mmol/L NaCl, 1.4 mmol/L phosphate, 10.9 mmol/L trisodium citrate, pH 7.4). After centrifugation for 30 minutes at 18,890x g and 20°C, 225 μL of the supernatant was removed again. The MP pellet was then resuspended with 75 μL PBS-citrate. In addition, in a subset of samples MPs were isolated as described by Shet et al. because those authors also studied MPs in human SCD.7

**Flowcytometry**

Five μL of the MP suspension was diluted in 35 μL CaCl$_2$ (2.5 mmol/L)-containing PBS. Then 5 μL APC-labeled annexin V was added to all tubes plus 5 μL of the cell-specific monoclonal antibody or isotype-matched control antibodies. The samples were incubated in the dark for 15 minutes at room temperature. After incubation, 900 μL of calcium-containing PBS was added to all tubes (except to the annexin V control, to which 900 μL citrate-containing PBS was added). Samples were analyzed for one minute in a fluorescence automated cell sorter (FACS Calibur) with CellQuest software (Becton Dickinson, San Jose, CA). Both forward scatter (FSC) and sideward scatter (SSC) were set at logarithmic gain. MPs were identified on basis of their size and density and on their ability to bind cell-type specific CD antibodies and annexin V.6 Annexin V measurements were corrected for auto-fluorescence. Labeling with cell-specific monoclonal antibodies was corrected for identical concentrations of isotype-matched control antibodies by subtracting the amount of isotype-matched positive events from the total positive events.

**Thrombin generation**

The thrombin generation test (TGT) was used as described previously.16 Briefly, MPs were reconstituted in defibrinated (reptilase-treated) normal pool (MP-free) plasma. For the inhibition experiments, the defibrinated plasma and the MPs were separately incubated for 30 minutes at ambient temperature with 20 and 5 μL of antibodies against coagulation factors VII or XI, or tissue factor pathway inhibitor (TFPI), respectively. Anti-factor VII was used to inhibit the extrinsic pathway and anti-factor XI to inhibit the intrinsic pathway and the fakctor XI-dependent amplification loop. Plasma and MPs were pooled after preincubation and incubated for an
additional 10 minutes at 37°C. Thrombin generation was started ($t = 0$) by addition of 30 μL CaCl₂ (16.7 mmol/L final concentration). At fixed intervals, 3 μL- aliquots were removed and added to 147 μL pre-warmed chromogenic substrate Pefachrome TH-5114 (Pentapharm, Basel, Switzerland) to measure the concentration of free thrombin. After 3 minutes, 90 μL 1 mol/L citric acid was added to stop the conversion of Pefachrome TH-5114. The generated amount of p-nitroaniline was determined at $\lambda = 405$ nm with a Spectramax microplate reader (Molecular Devices, Union City, CA). For quantitative analysis, the results were expressed as the area under the thrombin generation curve (AUC), calculated for the time interval between 0 and 15 minutes after addition of CaCl₂.

**Statistics**

Continuous data were expressed as medians with corresponding inter-quartile ranges (IQR). Between group differences were tested with the Mann-Whitney U test or Wilcoxon rank test in case of paired analysis. Categorical data are presented as percentages or numbers. Differences between groups of categorical data are tested with the Chi-square test. For correlation studies the Spearman Rank correlation coefficient was determined. To analyze data for possible confounding by multiple testing errors, correlations were also analyzed in mixed models with the patient as subjects. Results of these tests are only mentioned, when different to the described results. P-values $\leq 0.05$ were considered statistically significant. Statistical analysis was performed by using SPSS 12.0.2 (SPSS Inc, Chicago, IL).
Results

Patients
A total of 25 consecutive patients with a painful crisis were included, with 13 also providing baseline samples. For patient characteristics see Table 1. The median duration of hospital admission was eight days and none of the patients developed complications such as an acute chest syndrome, sepsis or renal failure.

<table>
<thead>
<tr>
<th>Table 1. Patient Characteristics.</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>Female (%)</td>
<td>56</td>
<td>54</td>
</tr>
<tr>
<td>Age (years)</td>
<td>31(26-41)</td>
<td>28(21-38)</td>
</tr>
<tr>
<td>Genotype*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbSS</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Sδ0-thal</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Sβ+ -thal</td>
<td>5</td>
<td>3</td>
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<tr>
<td>HbsC</td>
<td>8</td>
<td>3</td>
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<tr>
<td>Blood parameters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (mmol/L)</td>
<td>6.0 (5.1-6.5)</td>
<td>5.8 (5.6-6.5)</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>5.3 (4.3-6.0)</td>
<td>7.6 (3.3-9.8)</td>
</tr>
<tr>
<td>Thrombocytes (x 10⁹/L)</td>
<td>273 (97-407)</td>
<td>239 (154-332)</td>
</tr>
<tr>
<td>Leukocytes (x 10⁹/L)</td>
<td>9.8 (9.0-13.8)</td>
<td>8.2 (5.3-12.0)</td>
</tr>
<tr>
<td>Lactate Dehydrogenase (U/L)</td>
<td>374 (288-457)</td>
<td>287 (231-454)</td>
</tr>
</tbody>
</table>

*Number of patients with specified genotype.

Numbers and origin of microparticles
Median (inter-quartile range) numbers of MPs during painful crisis and steady state are depicted in Table 2. In steady state as well as during crisis, the majority of MPs originated from platelets (CD61+) and erythrocytes (glycophorin A+ (CD235+)). A distinct subset of transferrin receptor (CD71+)-exposing MPs was present, but neither MPs originating from monocytes (CD14+) nor endothelial cells (CD144+, CD146+, CD62E+) were detectable, and also no MPs exposing TF could be identified.

The total numbers of circulating MPs were not correlated to any of the parameters reflecting hemolysis (LDH and hemoglobin), endothelial activation (vWF-ag), fibrinolysis (D-dimer) or coagulation activation (F1+2c). The platelet-derived MPs (CD61+) represented the largest population of MPs in this study and correlated strongly with the number of circulating platelets,
but no correlations were found with hemoglobin, LDH, or specific markers (Table 3). Subpopulations of MP from activated platelets, i.e. MPs exposing CD61 plus either P-selectin (CD62P) or CD63, did not differ significantly between baseline and painful crisis (P=0.44 and P=0.51, respectively).

The second largest population of MPs found in this study were the erythrocyte-derived MPs (CD235+), which correlated with LDH (r=0.59, P<0.001) and Hb (r=-0.58, P<0.001; Table 3). Erythrocyte-derived MPs were also associated with markers of the in vivo coagulation and fibrinolysis activation status as well as endothelial activation (Table 3; Figure 1). CD71-positive MPs did not stain for glycophorin A and were associated with both the number of circulating reticulocytes as well as platelets (Table 3).

### Table 2. Microparticle numbers and markers of blood activation during painful crisis and baseline conditions.

<table>
<thead>
<tr>
<th></th>
<th>Painful crisis</th>
<th>Baseline</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPs (x 10^6/ml)</td>
<td>5.5 (2.9-9.6)</td>
<td>6.1 (4.0-7.7)</td>
<td>0.47</td>
</tr>
<tr>
<td>Percentage of MPs (%) positive for:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD71</td>
<td>5.1 (2.6-7.4)</td>
<td>5.0 (1.9-5.7)</td>
<td>0.36</td>
</tr>
<tr>
<td>GlycoA</td>
<td>9.7 (4.3-15.3)</td>
<td>4.8 (3.2-7.1)</td>
<td>0.18</td>
</tr>
<tr>
<td>CD61</td>
<td>86.1 (78.8-91.0)</td>
<td>91.4 (89.6-93.0)</td>
<td>0.09</td>
</tr>
<tr>
<td>Markers of coagulation activation:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1+2 (pmol/L)</td>
<td>307 (214-565)</td>
<td>213 (151-418)</td>
<td>0.06</td>
</tr>
<tr>
<td>D-dimer (μg/L)</td>
<td>2053 (911-3834)</td>
<td>1093 (599-2013)</td>
<td>0.04</td>
</tr>
<tr>
<td>vWF-ag (%)</td>
<td>193 (168-247)</td>
<td>141 (117-155)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*Corrected for number of events with isotype controls.

### Table 3. Correlations between blood parameters, markers of blood activation and numbers of microparticles.

<table>
<thead>
<tr>
<th></th>
<th>MP stained for:</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>GlycoA</td>
<td>CD61</td>
<td>CD71</td>
</tr>
<tr>
<td>Hematological parameters:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hemoglobin (mmol/L)</td>
<td>-0.58**</td>
<td>0.08</td>
<td>-0.15</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>0.59**</td>
<td>-0.17</td>
<td>0.05</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>0.32</td>
<td>0.40*</td>
<td>0.76**</td>
</tr>
<tr>
<td>Platelets (x 10^9/L)</td>
<td>0.47*</td>
<td>0.65**</td>
<td>0.62**</td>
</tr>
<tr>
<td>Markers of coagulation activation, fibrinolysis and endothelial activation:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vWF-ag (%)</td>
<td>0.44**</td>
<td>-0.07</td>
<td>0.00</td>
</tr>
<tr>
<td>F1+2 (pmol/L)</td>
<td>0.53**</td>
<td>-0.07</td>
<td>-0.02</td>
</tr>
<tr>
<td>D-dimer (μg/L)</td>
<td>0.52**</td>
<td>-0.01</td>
<td>0.14</td>
</tr>
</tbody>
</table>

All values are Spearman correlation coefficients. *P<0.05 **P<0.005
When MPs were isolated as performed previously by Shet and coworkers, the total number of “events” detected by flowcytometry decreased substantially. By far most of these “events” did not stain for any of the antibodies tested. As a consequence, the percentages of MPs staining for any of the tested antibodies were low thereby increasing the percentage false positives. This resulted a very low yield of MPs (percentage Annexin V positive small particles) and the percentage of MPs staining positive for isotype (negative) controls (eg: FITC-labelled IgG_{1} and IgG_{\text{2b}}, PE) was high. (see supplemental data) Even when using their isolation protocol, we were still unable to specifically detect MPs originating from either monocytes or endothelial cells, or exposing TF (data not shown).

![Image](image_url)

**Figure 1. Correlations between glycophorin A+ MPs and blood parameters.**

Correlations between glycophorin A+ MPs and markers of in vivo endothelial activation (vWF-Ag), fibrinolysis (D-dimer), and coagulation activation (F_{1+2}). Y- and X-axes are logarithmic. **P < 0.005.

<table>
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<th>Additional data table 1. Results using different isolation protocols.</th>
<th>Centrifuge speed</th>
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<tr>
<td>N</td>
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<tr>
<td>Events in the MP-gate (10^6/ml)</td>
<td>8.1 (5.7-10.1)</td>
</tr>
<tr>
<td>MPs* (10^6/ml)</td>
<td>6.1 (4.0-7.7)</td>
</tr>
<tr>
<td>Events in MP-gate positive for Annexin V(%)</td>
<td>78.8 (72.3-84.4)</td>
</tr>
<tr>
<td>Number of MPs (10^6/ml) positive for**:</td>
<td></td>
</tr>
<tr>
<td>CD61</td>
<td>5.45 (2.88-7.06)</td>
</tr>
<tr>
<td>GlycoA</td>
<td>0.33 (0.30-0.43)</td>
</tr>
<tr>
<td>CD71</td>
<td>0.24 (0.15-0.30)</td>
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</tbody>
</table>

*Microparticles (MPs) are defined as Annexin V positive events in the MP-gate. **Corrected for isotype controls.
Thrombin generation

The results of the thrombin generation assays are depicted in Figure 2. The AUC of the thrombin generation curve, representing the total amount of thrombin generated, correlated with the total number of circulating MPs (R=0.63, P<0.001). Thrombin generation was unaffected by pre-incubation with anti-human factor VII but increased slightly in the presence of anti-TFPI (16%; P=0.01). In contrast, in the presence of anti-factor XI, thrombin generation decreased about 2-fold (P<0.001). The extent of this inhibition was significantly associated with numbers of erythrocyte-derived MPs (r=0.50, P=0.023), but not with platelet-derived MPs or reticulocyte-derived MPs (r=-0.03 and r=-0.10, respectively; P=NS). Also the absolute difference in thrombin generation (ΔAUC) between the experiments with and without factor XI antibody correlated with the absolute number of glycoporphin A+ MPs (r=0.55, P=0.002; Spearman, Figure 3).

![Graph](image)

**Figure 2 & 3. Thrombin generation and correlation with Glycoporphin A+ MPs and anti-human factor XI effect.**

Figure 2: The gray (normal) bar shows the median (error bar: 75th quartile) of thrombin generation expressed as AUC after reconstitution of MPs isolated from patient blood to defibrinated and MP-free normal pool plasma. The “aTFPI”, “aXI” and the “aVII” bars show the effects of the indicated antibodies on MP-induced thrombin generation. Figure 3: Correlation between the total number of glycoporphin A+ MPs and the extent of inhibition of thrombin generation by anti-human factor XI (r=0.55 P=0.002). Y- and X-axes are logarithmic.
Discussion

We analyzed the origin of circulating microparticles in patients with SCD during painful crises and steady state and studied their relationship with in vivo coagulation activation, hemolysis, fibrinolysis and endothelial activation.

First, we demonstrated that almost all circulating MPs were derived from erythrocytes and platelets, and that the total number of MPs did not differ significantly between baseline conditions and crisis, although a shift towards more erythrocyte-derived MPs was observed during the painful crisis. These data are in line with previous observations that high numbers of both erythrocyte-derived MPs and platelet-derived MPs are present in patients with SCD. Furthermore, we identified a distinctive population of MPs exposing CD71, the transferrin receptor, but lacking glycoporphin A. These CD71+ MPs were highly associated with the number of circulating reticulocytes and may reflect the shedding of the transferrin receptor during reticulocyte maturation. Apart from these MPs, no other populations of circulating MPs could be identified in the patient plasma samples. In particular, no monocyte-derived (CD14+) or endothelial cell-derived (CD144+, CD146+, CD62E+) MPs could be identified. Also, no MPs exposing TF were detectable in our fractions. These findings are in contrast with previous reports, which showed substantial populations of both endothelial- and monocyte-derived MPs exposing TF in sickle cell patients. Possibly, these differences are explained by the much higher centrifugation forces used to isolate cell-derived vesicles as compared to our study and other investigators. When we used this 100,000 x g isolation protocol, we not only had a relatively low yield of cell-derived vesicles, but also did not observe MPs from monocytes or endothelial cells, or staining for TF, when corrected for control antibody binding.

While no correlation was observed between the total number of circulating MPs and coagulation activation, erythrocyte-derived MPs proved to be specifically related with in vivo coagulation, fibrinolysis and endothelial activation. These observations confirm previous studies of patients with thalassemia and PNH, pointing towards a direct relation between hemolytic anemia and the hypercoagulability. Similar to our observation, Setty et al. demonstrated that only the number of phosphatidylserine (PS)-exposing erythrocytes correlated with in vivo markers of endothelial activation, fibrinolysis and coagulation activation, whereas this relation was absent with PS-exposing platelets. Moreover, a recent study confirmed that erythrocyte-derived MPs are associated with hemolysis-related coagulation activation.

In our thrombin generation experiments, we observed an almost 50% reduction in thrombin generation by anti-human factor XI. Factor XI plays an important role in enhancing thrombin generation, since trace amounts of thrombin can activate factor XI to factor X, which then augments thrombin generation via the tenase complex. Because the factor VII / tissue factor complex, i.e. initiation of the coagulation process, is evidently not involved, we presume the factor
XI-supported amplification by phosphatidyserine exposure on erythrocyte-derived MPs to be involved.

Our present results do not exclude that small numbers of TF-exposing MPs are present in the plasma samples of SCD patients, since thrombin generation by isolated fractions of MPs from these patients was enhanced when TFPI was blocked. Nevertheless, the amount of TF present in such MPs was insufficient to trigger TF/VII-dependent coagulation activation in normal plasma, i.e. plasma containing physiologically levels of TFPI. We can not exclude that inhibition of protein S, a recently described cofactor for TFPI, may reveal the true activity of MP-associated TF under these conditions.24

From our present study, we conclude that the procoagulant state in SCD is, at least in part, due to the procoagulant effects of circulating erythrocyte-derived MPs. Their relation with activation of factor XI and the ability of anti-factor XI to block thrombin by MPs isolated from plasma samples of SCD patients suggests an important role of factor XI-dependent thrombin generation in these patients.
Reference List

Sickle cell patients are characterized by a reduced glycocalyx volume
(Based on: Haematologica. 2008 Feb;93(2):307-8)

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² Department of Internal Medicine, Slotervaart Hospital, Amsterdam.
³ Department of Vascular Medicine, Academic Medical Center, Amsterdam.
⁴ Immunology Department, Red Cross Blood Bank Foundation, Curaçao, Netherlands Antilles,
⁵ Department of Hematology, Erasmus Medical Centre, Rotterdam.
Abstract

Recently, the importance of the glycocalyx as a protective anti-inflammatory and anti-adhesive barrier at the luminal side of endothelial cells has been established. The mean glycocalyx volume was significantly reduced in patients with HbSS/HbSβ0 (median 0.47L, inter quartile range 0.27-0.66) and HbSC/HbSβ+ (0.23L, 0.0-0.58) as compared to controls (1.09L, 0.52-1.77) (p=0.03).
Background and objectives

The vaso-occlusive process of sickle cell disease (SCD) leads to accumulating ischemic organ damage and a decreased life expectancy. Vaso-occlusion in SCD was primarily attributed to the mechanical obstruction of the microvasculature by circulating sickled erythrocytes. Over the last decades, it has become clear that endothelial activation and dysfunction play a central role in sickle cell vaso-occlusion. Hypoxia, pro-inflammatory cytokines, thrombin, reactive oxygen species, activated leukocytes, as well as direct mechanical injury by sickled erythrocytes all induce endothelial activation in SCD. Activated endothelium itself contributes to the initiation and progression of vaso-occlusion by releasing pro-inflammatory cytokines, pro-coagulant microparticles and by initiating coagulation with surface tissue factor expression. Endothelial activation is characteristic of SCD, even in the clinically asymptomatic state, with further increments during acute vaso-occlusive complications. Furthermore, the extent of endothelial activation was recently demonstrated to be related to the presence of severe disease related manifestations such as pulmonary hypertension. New insights into endothelial biology have demonstrated that, in the quiescent state, the endothelium is shielded from circulating blood cells and proteins by the glycocalyx, a highly hydrated cell free mesh of membrane-associated proteoglycans, glycosaminoglycans, glycoproteins and glycolipids located at the endothelial surface. With a thickness ranging from 0.5 to 3.0 µm, it exceeds the intra-luminal size of most endothelial adhesion molecules, thereby potentially preventing interactions of the endothelium with blood constituents. The glycocalyx volume is regulated by numerous factors and rapid degradation has been observed during ischemia, hypoxia, and exposure to both tumor necrosis factor alpha and oxidized low density lipoproteins. Therefore, in conditions of ischemia and inflammation, which occur continuously in SCD, a reduced glycocalyx volume may facilitate interactions of activated endothelial cells with blood cells and proteins. Given the important role of endothelial activation in the pathophysiology of SCD related vaso-occlusion, we set out to investigate whether the glycocalyx volume is reduced in patients with SCD.
Design and methods

Patients and controls
Consecutive adult sickle cell patients (HbSS, HbSβ0/+thalassemia or HbSC, confirmed with high performance liquid chromatography) visiting the out-patient clinic of the Academic Medical Center in Amsterdam were eligible for inclusion in this study. Exclusion criteria were a history of an acute vaso-occlusive episode (painful crisis, acute chest syndrome, stroke, splenic- or liver sequestration) or blood transfusion 4 weeks prior to sample collection; diabetes mellitus; hemorrhagic retinopathy; hemorrhagic disorders or hypertension (systolic and diastolic blood pressure >140 mm Hg or >90 mm Hg, respectively). For data analysis patients with the most severe genotypes (HbSS and HbSβ0-thalassemia) were grouped together, as were patients with the relatively milder genotypes (HbSC and HbSβ+ -thalassemia). Age, sex and race matched individuals heterozygous for hemoglobin C or S (HbAS and HbAC, confirmed by high performance liquid chromatography) served as healthy controls. All patients and controls gave written informed consent and this study was approved by the Medical Ethics Committee of the Academic Medical Center in Amsterdam. The study was carried out in accordance with the principles of the Declaration of Helsinki.

Measurement of systemic glycocalyx volume
The glycocalyx volume can be estimated by comparing circulating blood volume with the intravascular distribution volume of a glycocalyx-permeable tracer such as neutral dextran 40 (molecular weight 40 kDa) as has been described previously. To determine the intravascular distribution volume of dextran 40, the concentration of dextran 40 at the time of injection was estimated by exponential fitting of the measured dextran 40 concentrations. The intravascular distribution volume of labeled, autologous erythrocytes was used to quantify circulating blood volume. Blood was drawn into heparin collector tubes and centrifuged at 1,330 rpm for 5 min. Subsequently, 250 mg/ml of sodium fluorescein was added to the erythrocyte fraction for 5 min. After washing with water (5% natrium chloride), labeled erythrocytes were re-suspended in saline to the initial volume and re-infused into the patients/controls. Subsequently, blood was drawn at 4, 5, 6, and 7 min after infusion. The fraction of labeled erythrocytes compared with total erythrocyte pool was used to estimate circulating erythrocyte volume. Pre-injection unlabeled erythrocytes (t = –1) served as negative controls. Labeled erythrocytes were measured using a FACScan (FACSCalibur; Becton Dickinson, Mountain View, CA), during which at least 100,000 cells were counted to measure the circulating fraction of labeled erythrocytes. Circulating plasma volume was calculated from circulating erythrocyte volume (Vrbc) and systemic hematocrit (Hsys) by the following formula: circulating plasma volume = ([1 – Hsys] x Vrbc)/Hsys.1016
Dextran 40 was used as a probe to estimate total intravascular volume including the glyocalyx compartment.\textsuperscript{15,17,20} A bolus of 10 ml dextran 1 (Promiten; NPBI International, Emmercompascuum, the Netherlands) was injected to attenuate the risk for anaphylactic reactions. At least 1 h later, 100 ml dextran 40 (Rheomacrodex; NPBI International) was administered intravenously, followed by repeated blood sampling at 5, 7, 10, 15, 20, and 30 min. The dextran 40 concentration was calculated by measuring the increase in glucose concentration in the post infusion samples after hydrolyzation of dextran 40 glucose polymers.\textsuperscript{20} The glucose concentration per time point was assessed in duplicate using the hexokinase method (Gluco-quant, Hitachi 917; Hitachi). To determine the intravascular distribution volume of dextran 40, the concentration of dextran 40 at the time of injection was estimated by exponential fitting of the measured dextran 40 concentrations. To further evaluate the relation between glyocalyx volume and other parameters the groups of patients were split into halves; a small glyocalyx volume-group and a large glyocalyx volume-group.

Analysis of blood samples
At baseline, blood samples were collected and placed on ice immediately. Hematocrit was measured after centrifugation of heparinized blood at 10,000 rpm for 5 minutes. The remaining blood samples were centrifuged at 3,000 rpm for 15 minutes; plasma was collected and stored at -80°C. Quantitative plasma hyaluronan levels were measured by enzyme-linked immunosorbent assay (Echelon Biosciences, Salt Lake City, UT). Plasma hyaluronidase levels were determined with a previously described assay (intersample coefficient of variation <20%).\textsuperscript{21}

Statistics
Data were expressed as medians with corresponding interquartile ranges (IQR) or as stated otherwise. Between group differences were tested with the Kruskal Wallis Test and post-tested to find which groups differ from each other with Mann-Whitney U test. Categorical data are presented as percentages or numbers. Differences between groups of categorical data are tested with the Chi-square test. For correlation studies the Spearman Rank correlation coefficient was determined. Multiple regression analysis with genotype as co-factor was used to correct for genotype. P-values ≤0.05 were considered statistically significant. Statistical analysis was performed by using SPSS 12.0.2 (SPSS Inc, Chicago, IL).
Results

Patients and controls
Twenty sickle cell patients and 10 individuals with the HbAS (n=9) or HbAC (n=1) were included. The severe genotype group consisted of 9 HbSS and 3 HbSβ-thalassemia patients, and the mild genotype group consisted of 6 HbSC and 2 HbSβ+-thalassemia patients. The baseline patient characteristics and laboratory data are presented in Table 1. Four patients in the severe genotype group used hydroxyurea.

<table>
<thead>
<tr>
<th>Table 1. Baseline Characteristics.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristics</td>
</tr>
<tr>
<td>N</td>
</tr>
<tr>
<td>Age (y)</td>
</tr>
<tr>
<td>Male/Female</td>
</tr>
<tr>
<td>Systole (mmHg)</td>
</tr>
<tr>
<td>Diastole (mmHg)</td>
</tr>
<tr>
<td>BMI</td>
</tr>
</tbody>
</table>

Blood parameters
- Hemoglobin (mmol/L) 5.5 (5.1-6.0) 7.1 (6.3-7.8)* 8.5 (7.3-8.9)**
- Reticulocytes (%) 8.8 (6.3-11) 2.8 (2.1-2.9)** 1.1 (0.9-1.7)**
- Leucocyte count (x10⁹/L) 8.0 (6.5-10.1) 7.0 (5.7-8.2) 7.2 (3.3-9)
- Lactate dehydrogenase (U/L) 385 (289-570) 220 (181-235)** 171 (164-181)**
- Fetal Hemoglobin (%) 9.4 (4.2-14.7) 2.4 (0.5-3.6) 0.5 (0.5-0.5)**

All numbers are medians (interquartile range). *P<0.05 versus HbSS/HbSβ*-thal ** P<0.001 versus HbSS/HbSβ*-thal. *P* values are based on Mann Withney U or Chi-square tests.

Glycocalyx volume
The glycocalyx volume was statistically significantly lower in patients as compared to controls (Figure; Table 2). Hyaluronan levels, but not hyaluronidase activity, differed significantly between groups (Table 2). There were no statistically significant differences in blood volume, plasma volume and dextran distribution volume between the groups (data not shown). Analysis of bi-variate correlations (Spearman) of all data showed that glycocalyx volume was correlated to plasma levels hyaluronidase (r= -0.431; P=0.017). Correction for genotype did not alter this relation. Use of hydroxyurea did not have influence on glycocalyx volume.
Figure. Glycocalyx volume in sickle cell patients and healthy controls.
The glycocalyx volume differed significantly between the HbSS/HbSβ0-thal group (median 0.47 liters (L), IQR 0.27-0.66), the HbSC/HbSβ+-thal group (0.23L, IQR 0.0-0.58) and healthy controls (1.09L, IQR 0.52-1.77) (Kruskal Wallis P=0.025). Bars represent medians and error bars the 75th percentile.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HbSS/HbSβ0-thal</th>
<th>HbSC/HbSβ+-thal</th>
<th>Controls</th>
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<tbody>
<tr>
<td>Glycocalyx volume (L)</td>
<td>0.47 (0.27-0.66)</td>
<td>0.23 (0.0-0.58)</td>
<td>1.09 (0.52-1.77)*</td>
</tr>
<tr>
<td>Hyaluronan (mg/mL)</td>
<td>170 (129-299)</td>
<td>96 (89-109)*</td>
<td>120 (84-184)</td>
</tr>
<tr>
<td>Hyaluronidase (U/ml)</td>
<td>118 (70-188)</td>
<td>108 (81-145)</td>
<td>106 (91-242)</td>
</tr>
</tbody>
</table>

All numbers are medians (interquartile range). *P<0.05 versus HbSS/HbSβ0-thal (Mann Withney U test).
Interpretation and conclusion

New insights into endothelial biology have demonstrated an important role of the glycocalyx in vascular homeostasis. Loss of glycocalyx by acute hyperglycemia in healthy controls results in endothelial dysfunction and glycocalyx loss is associated with microvascular damage in type 1 diabetes. In the present study, we demonstrated that the glycocalyx volume is significantly reduced in patients with SCD as compared to the controls (the latter characterized by glycocalyx volumes comparable to those reported for healthy controls in other studies). The glycocalyx volume in our sickle cell patients was comparable to that reported in patients with diabetes mellitus type 1. The median glycocalyx volume in the HbSS/HbSβ0-thal group (0.47L) was slightly higher than the median glycocalyx volume (0.23L) in the HbSC/HbSβ+ -thal group. This difference was not significant and might be caused by the low number of subjects in the HbSC/HbSβ+ -thal group or the fact the we may have included patients with HbSC/HbSβ+ -thal with relatively severe disease.

Hyaluronidase is an enzyme that degrades hyaluronan, the major glycosaminoglycan component of the glycocalyx. In line with the findings in patients with diabetes type 1, glycocalyx volumes were inversely correlated to hyaluronidase levels in our patients. However, as the hyaluronidase levels were comparable between the different groups of patients and controls this can not explain the difference between these groups in glycocalyx volume. Studies in humans and animals have shown that the glycocalyx volume is reduced after episodes of experimental ischemia, hypoxia and after exposure to oxidized low-density lipoproteins and oxygen radicals. Acute hyperglycemia, which is associated with the formation of reactive oxygen species, results in a profound loss of glycocalyx volume in healthy volunteers. Continuous tissue ischemia and reperfusion is associated with the formation of reactive oxygen species in SCD which may result in a reduced glycocalyx volume.

A reduction of the glycocalyx could have several important consequences in SCD. Adhesive interactions between sickle red blood cells, leukocytes and the activated endothelium play a pivotal role in the initiation and propagation of SCD related vaso-occlusion. A reduced glycocalyx volume may facilitate such interactions. A reduced glycocalyx volume may also contribute to the hypercoagulable state of SCD by reducing the availability of naturally occurring anticoagulants, such as thrombomodulin and antithrombin, at the vessel wall.

In conclusion, we demonstrate that the presented sickle cell patients are characterized by a reduced glycocalyx volume. We hypothesize that glycocalyx perturbation could be a new factor of bidirectional importance in the complex pathophysiology of SCD, as glycocalyx volume reductions can both result from and contribute to vaso-occlusion.
Acknowledgments

E.J. van Beers performed the research, analyzed the data and wrote the paper. M.Nieuwdorp designed and performed the research and critically reviewed the paper. L.M.Evers and A.J.Duits performed the research and critically reviewed the paper. H.Vink designed the research, analyzed the data and critically reviewed the paper. J.J.B.Schnog analyzed the data and critically reviewed the paper. B.J.Biemon designed the research, analyzed the data and wrote the paper.
Reference List


Part III:
Pulmonary hypertension
Large and medium sized pulmonary artery obstruction does not play a role of primary importance in the etiology of sickle cell disease associated pulmonary hypertension

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Abstract

Pulmonary hypertension (PHT) occurs in approximately 30% of adult sickle cell patients and is a risk factor for early death. The potential role of pulmonary artery obstruction, whether due to emboli or in situ thrombosis, in the etiology of sickle cell disease (SCD) related PHT is unknown. Consecutive sickle cell patients were screened for PHT (defined as a tricuspid regurgitant jet flow velocity ≥2.5m/s) employing echocardiography and were evaluated for pulmonary artery obstruction with ventilation-perfusion (VQ) scintigraphy.

Fifty-three HbSS, 6 HbSβ0-thalassemia, 20 HbSC and 6 HbSβ+-thalassemia patients were included. The overall prevalence of PHT was 41% in HbSS/HbSβ0-thal patients and 13% in HbSC/HbSβ+-thal patients. High probability VQ-defects (PIOPED criteria) were detected in 2 patients, one of whom had PHT. In HbSS/HbSβ0-thal patients with PHT, 19 (86%), 2 (9%) and 1 (5%) had low, intermediate or high-probability scans as compared to 30 (97%), 1 (3%) and 0(0%) in HbSS/HbSβ0-thal patients without PHT (p=0.31). In HbSC/HbSβ+-thal patients with PHT, 3(100%), 0(0%) and 0(0%) had low, intermediate and a high-probability scans as compared to 19(90%), 1(5%), and 1 (5%) in HbSC/HbSβ+-thal patients without PHT (p=0.86). There were no statistical differences in irregular distribution of the radiopharmaceutical or non-specific signs associated with PHT between patients with and without PHT.

Although small pulmonary artery obstruction cannot be excluded, large to medium sized pulmonary artery obstruction is an unlikely primary causative factor in SCD-related PHT.
Introduction

Pulmonary hypertension (PHT) is a recognized complication of sickle cell disease (SCD) occurring in approximately 30% of adult patients with sickle cell anemia. Once manifest, it is associated with an increased risk of early death. The pathophysiology of SCD related PHT is not completely elucidated, but reduced nitric oxide availability due to chronic hemolysis is considered to be of major importance. However, other factors, such as in situ thrombosis and/or pulmonary embolism (referred to as pulmonary artery obstruction from here on) may also be at play. SCD, as well as other forms of hemolytic anemia (such as thalassemia), are characterized by a hypercoagulable state, and it is increasingly recognized that PHT occurs in chronic hemolytic anemias other than SCD. Several small autopsy studies have shown pulmonary artery obstruction to occur in sickle cell patients, with a large landmark autopsy study demonstrating pulmonary artery obstruction in 16% of HbSS patients. Furthermore, large pulmonary artery obstruction has been reported as the cause of PHT in two sickle cell patients. Also, in a retrospective study, Stein and colleagues have recently shown that pulmonary embolism and/or in situ thrombosis seems to occur more frequently in sickle cell patients as compared to matched controls without SCD. Pulmonary artery obstruction could therefore play a role of importance in the development SCD-related PHT as described in other forms of PHT. If pulmonary artery obstruction plays a role in the pathophysiology of SCD related PHT, early recognition would be of cardinal importance to institute potential therapies such as anticoagulation as early as possible. Ventilation and perfusion (VQ) scintigraphy is an accurate and recommended method to screen for pulmonary artery obstruction in the diagnostic work-up of PHT. By employing VQ-scintigraphy, we set out to investigate whether large to medium sized pulmonary artery obstruction occurs in consecutive ambulatory adult sickle cell patients, and if so, whether it is associated with PHT.
Methods

Patients
Consecutive sickle cell patients visiting the outpatient hematology/internal medicine clinics of the Academic Medical Center (Amsterdam, the Netherlands), the Slotervaart Hospital (Amsterdam, the Netherlands) and the Erasmus Medical Center (Rotterdam, the Netherlands) were eligible for this study. Inclusion criteria were: high performance liquid chromatography confirmed diagnosis of SCD, age 18 years and older and written informed consent. Exclusion criteria were: known congestive heart failure, known chronic obstructive pulmonary disease or poorly controlled asthma, pregnancy and a painful crisis in the preceding 2 weeks (as pulmonary artery pressure increases during a painful crisis\textsuperscript{15}) and/or an acute chest syndrome in the preceding three months before study entry. The patients’ medical histories were studied by chart review. Acute chest syndrome was defined as the finding of a new pulmonary infiltrate on chest radiography in combination with fever, respiratory symptoms, or chest pain for which medical treatment was needed.\textsuperscript{16} The protocol was reviewed by a central medical ethical committee (Slotervaart Hospital) and subsequently reviewed and approved in the Academic Medical Center and Erasmus Medical Center. The study was carried out in accordance with the principles of the Declaration of Helsinki.

Trans-thoracic echocardiography
Trans-thoracic echocardiography was performed using conventional clinical echocardiographic equipment with 2.5- or 3.5-MHz transducers. Trans-thoracic M-Mode, Doppler and two-dimensional images were obtained from para-sternal long- and short-axis, apical four- and two chamber, and subcostal four-chamber views. Echocardiograms were reviewed to assess the pericardium, valvular anatomy and function, left- and right-sided chamber size, cardiac function and the presence of shunts. Left ventricular diastolic function was assessed by transmitral inflow and pulmonary vein flow signal analysis. Tricuspid regurgitant jet flow velocity (TRV) was identified by color flow Doppler techniques and maximum jet velocity was measured and recorded. Right ventricular systolic pressure was estimated based on the modified Bernoulli equation and considered to be equal to the systolic pulmonary artery pressure (sPAP) in the absence of right ventricular outflow obstruction. This has been validated to correlate with pulmonary artery systolic pressure in the absence of right ventricular outflow obstruction and pulmonary stenosis in patients with SCD.\textsuperscript{1} PHT was defined as a TRV $\geq$ 2.5 m/s. Pulmonary-artery pressures were considered normal in patients with trace or no tricuspid regurgitation (with the TRV assigned a value of 1.3 m/s in the latter group).\textsuperscript{1} Diastolic dysfunction was defined as an E/A < 1.0.
VQ-scintigraphy
VQ-scintigraphy was performed with 100-200 MBq of technetium-99m macroaggregated albumin particles (Mallinckrodt, Petten, The Netherlands) in all participating centers. Images were acquired in six views (500 kilocounts per view, 256 matrix). Krypton-81m gas was used for ventilation scintigraphy acquiring the same six views as in the perfusion scintigraphy (500 kilocounts per view, 256 matrix). VQ-scans were anonymously analyzed in PAX-2000 by two trained observers who were blinded to genotype and echocardiographic findings. The VQ-scans were analyzed according to the Prospective Investigation of Pulmonary Embolism Diagnosis (PIOPED) criteria for the diagnosis of pulmonary embolism.\textsuperscript{17} Perfusion abnormalities with an evident extrapulmonary cause, e.g. cardiomegaly or lung lobectomy were not scored as being perfusion defects. The probability scores normal, low and very low were combined in the category low probability.\textsuperscript{14} Next, a more detailed analysis was performed in which scintigraphic abnormalities were analyzed for each segment separately. Segments could be either normal, have an irregular distribution of the radiopharmaceutical (without perfusion defects) or could have a sub-segmental or segmental perfusion defect. In this analysis results on ventilation were disregarded. Lastly, all VQ-scans were analyzed in order to detect non-specific signs such as patchiness and diffuse left to right differences, which have been described in PHT patients.\textsuperscript{18} The medial basal segment was not scored because it is not recognizable on planar VQ-scintigraphy. Disagreement was solved by consensus between the two observers upon second review. To graph data, detailed scintigraphic abnormalities were summarized with a quantitative score: completely normal segments scored 0, segments with irregular distribution of the radiopharmaceutical (without defects) scored 1, sub-segmental defects scored 2, and segmental defects scored 4 points. Per patient all scored points were added up and divided by four to quantify the total lung area with perfusion defects. This score expresses the total abnormally perfused lung area in units equivalent to lung segments (segmental equivalents).

Statistics
All numbers are medians with corresponding inter-quartile range (IQR) unless stated otherwise. Difference in continuous data between groups was tested with the Mann-Whitney-U test. Difference in categorical data between groups was tested with the Chi-square test or the Fisher’s exact test when appropriate. For the analysis of perfusion defects as segmental equivalents ordinal categories were employed. For graphical summary of the data the difference between groups was calculated as difference in segmental equivalents. For correlation studies the Spearman rank correlation coefficient (\(r_s\)) was calculated. P-values \(\leq 0.05\) were considered statistically significant. Statistical analysis was performed by using SPSS 12.0.2 (SPSS Inc, Chicago, IL).
Results

Patients

Ninety consecutive patients were eligible for the study, 5 of whom declined participation. Eighty-five patients were included in the study. For data analysis, the more severe SCD genotypes, HbSS and HbSβ⁰-thalassemia, were grouped together, as were the relatively milder SCD genotypes HbSβ⁺-thalassemia and HbSC.¹⁹,²⁰ For patient demographics see Table 1.

Trans-thoracic echocardiography

Trans-thoracic echocardiography was performed in 78 of the 85 included patients as 7 patients failed to meet their appointment on several occasions. The overall prevalence of PHT was 32% (23 patients (41%) in the HbSS/HbSβ⁰-thal group and 3 patients (13%) in the HbSC/HbSβ⁺-thal group (P=0.018)). The TRV in sickle cell patients with PHT was: median 2.7m/s (IQR 2.6-2.8m/s), mean 2.7±0.2m/s, range 2.5-3.5m/s. The median sPAP was higher in the HbSS/HbSβ⁰-thal group as compared to the HbSC/HbSβ⁺-thal group. No difference in the prevalence of PHT was detected between male and female patients. No intra-cardiac shunts or right ventricular outflow obstruction were detected.

<table>
<thead>
<tr>
<th>Table 1. Patient characteristics.</th>
<th>HbSS (n=53)/HbSβ⁺-thal (n=6)</th>
<th>HbSC (n=20)/HbSβ⁺-thal (n=6)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>31 (21-47)</td>
<td>30 (24-39)</td>
<td>0.89</td>
</tr>
<tr>
<td>Sex (Males:females)</td>
<td>15:44</td>
<td>14:12</td>
<td>0.01*</td>
</tr>
<tr>
<td>History of ACS (%)</td>
<td>41.7%</td>
<td>8.3%</td>
<td>0.004*</td>
</tr>
<tr>
<td>Tricuspid regurgitant flow (m/s)</td>
<td>2.4 (1.6-2.6)</td>
<td>2.1 (1.3-2.3)</td>
<td>0.02</td>
</tr>
<tr>
<td>sPAP (mmHg)</td>
<td>28 (12-33)</td>
<td>23 (12-28)</td>
<td>0.04</td>
</tr>
<tr>
<td>Diastolic dysfunction (%)</td>
<td>8.3%</td>
<td>4.8%</td>
<td>1.00*</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>8.5 (7.4-9.4)</td>
<td>11.5 (10.6-12.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Leukocytes (x10⁹/L)</td>
<td>8.9 (7.2-10.5)</td>
<td>7.1 (4.6-9.4)</td>
<td>0.01</td>
</tr>
<tr>
<td>Fetal Hb (%)</td>
<td>8.6 (4.1-15.1)</td>
<td>1.0 (1.0-2.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>425 (344-647)</td>
<td>234 (214-299)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Numbers are medians (IQR). P-values are based on Mann-Whitney test. *P-value based on Chi-square test. ACS = acute chest syndrome, Hb = Hemoglobin, LDH = lactate dehydrogenase.

VQ scintigraphy

In total 83 patients underwent perfusion lung scanning. Two subjects repeatedly did not appear for their appointment and had no VQ-scintigraphy performed. All scans were of adequate quality for analysis.
The inter-observer overall agreement was 0.92 and the Kappa coefficient for sub-segmental and segmental defects versus none or any defect (high probability and intermediate) showed moderate agreement (Kappa = 0.45).

According to the PIOPED-criteria 49 (92%) patients had low probability VQ scans, 3 (6%) had intermediate probability scans and 1 (2%) had a high-probability scan in the HbSS/HbSβ0-thal group. In the HbSC/HbSβ+-thal group 22 (94%) patients had low probability VQ scans, 1 (3%) had an intermediate probability VQ scan and 1 (3%) had a high-probability VQ scan. Results of VQ scans analyzed with PIOPED criteria did not differ between the HbSS/HbSβ0-thal and HbSC/HbSβ+-thal groups (P=0.82).

Detailed analysis of scintigraphic abnormalities revealed 64 patients with no scintigraphic abnormalities, 4 patients with one or more segmental perfusion defects, 11 patients with one or more sub-segmental defects and 13 patients with one or more lung segments with irregular distribution of the radiopharmaceutical.

<table>
<thead>
<tr>
<th></th>
<th>HbSS/Sβ0-thal</th>
<th>HbSC/Sβ+-thal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHT</td>
<td>No PHT</td>
</tr>
<tr>
<td>High</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>2 (9%)</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>Low</td>
<td>19 (86%)</td>
<td>30 (97%)</td>
</tr>
</tbody>
</table>

Numbers are counts (percentages). *= P-value based on Chi-square test.

Figure 1. Difference in perfusion defects between patients with and without PHT.
Anterior and posterior view of the lung showing total difference in perfusion defects (segmental equivalents rounded to the nearest integer) between patients with PHT (N=25) and without PHT (N=52). The superior and postero-basal segment (of the lower lobe) of the left lung had significantly more perfusion defects in the PHT group compared to the non-PHT group (P=0.001 and P=0.018 respectively). The difference in perfusion of the apex of the right lung was not statistically significant (P=0.68). R=right; L=left; Segments are: Ap=apex; Ant=anterior; L=Lateral; M=Medial; SL=Superior lingual; IL= Inferior Lingula; S=superior; AB=Antero-basal; LB=Latero-basal; PB=Postero-basal.
The number of segments with irregular distribution of the radiopharmaceutical in the left lung was significantly higher than in the right lung (P=0.016). Neither a history of an acute chest syndrome nor the frequency of painful crisis was associated with high PIOPED probability score or with the detailed perfusion analysis score as described above (data not shown). The number of segments with irregular distribution of the radiopharmaceutical in the lower lobe and lingula of the left lung were significantly correlated to volume of the left ventricle end-diastolic volume ($r_s=0.293$; P=0.018). The presence of diastolic dysfunction was not related to perfusion defects (data not shown).

**Association of VQ-scintigraphy with PHT**

Six patients underwent VQ-scintigraphy but did not undergo a trans-thoracic echocardiography. All of these patients had low probability VQ scans according to the PIOPED criteria. In the remaining 77 patients, no statistical difference in pulmonary perfusion according to PIOPED criteria was observed between patients with or without PHT (see Table 2). A high probability VQ defect was detected in only 1 PHT patient. This patient had a history of an ACS complicated by a lung infarct. In the group of patients without PHT, a high probability VQ defect was detected in 1 patient with a history of pulmonary embolism. Two sickle cell patients with PHT had an intermediate probability VQ defect.

![Figure 2. Representative VQ-scans showing irregular distribution of the radiopharmaceutical.](image)

A. Anterior (ant), posterior (post), right posterior oblique (rpo) and left posterior oblique (lpo) view of a patient with a normal perfusion-scan. B. Anterior (ant), posterior (post), right posterior oblique (rpo) and left posterior oblique (lpo) view of a representative patient with irregular distribution of the radiopharmaceutical in the lower lobe of the left lung and evident cardiomegaly.
With detailed analysis, significantly more scintigraphic abnormalities were demonstrated in the superior and postero-basal segment of the left lower lobe in patients with PHT as compared to patients without PHT (P=0.001 and P=0.018 respectively) (see Figure 1). A separate analysis of the HbSS/HbSβ+thal patients demonstrated significantly more scintigraphic abnormalities in the superior (P=0.01) and postero-basal segment (P=0.047) of the left lower lobe in patients with PHT as compared to patients without PHT. In addition, more segments with irregular distribution of the radiopharmaceutical were seen in the lung segments of the left lung of the HbSS/HbSβ+-thal group with PHT (P=0.023) (Figure 2). This detailed analysis was not performed for the HbSC/HbSβ+-thal patients because of the low prevalence of PHT in this group. No differences were found in non-specific scintigraphic signs of PHT (such as patchiness and diffuse left to right differences) between the patients with and without PHT (data not shown). There was no statistically significant correlation between the TRV and presence of or number of perfusion defects (data not shown).

Twenty-one patients had only trace or no tricuspid regurgitation on trans-thoracic echocardiography. In this group, all 21 patients had low probability VQ-scans. Three patients had sub-segmental defects and the frequency of non-specific scintigraphic signs in this group was not significantly different from that of other patients without PHT (Figure 3).

![Perfusion defects per patient](image)

**Figure 3.** Total amount of perfusion defects per patient.

On the Y-axis the total amount of perfusion defects per patients is quantified in segmental equivalents (SE) as defined in the material and methods section. On the X-axis every individual patient is represented according to their respective TRV. Patients who did not undergo echocardiography are also shown. * All patients with trace or no tricuspid regurgitation with the TRV assigned 1.3 m/s. ** All patients with a TRV between 1.3 m/s and 2.5 m/s with the TRV increasing from left to right. # All patients with a TRV ≥ 2.5 m/s with the TRV increasing from left to right. There was no difference in the total amount of perfusion defects between the HbSS/HbSβ+-thal patients and the HbSC/HbSβ+-thal patients (data not shown).
Discussion

A recent landmark study demonstrated that PHT occurs in approximately 30% of adult sickle cell patients and carries a strongly increased risk of death as compared to sickle cell patients without PHT. Given this risk of early death, it is of paramount importance to identify treatable causes and/or aggravating factors of PHT in SCD. In this study, we investigated the potential role of pulmonary artery obstruction as a causative or contributing factor in sickle cell patients with PHT employing VQ-scintigraphy. VQ-scintigraphy was performed in 83 prospectively and consecutively included ambulatory adult sickle cell patients, making this the largest study to date regarding VQ scans in SCD. Even though the participating centers were referral hospitals for treating sickle cell patients, most sickle cell patients in the Netherlands are either cared for at referral centers or visit a referral center at regular intervals. We therefore feel that referral bias is limited. Furthermore, patient demographics and PHT prevalence were very similar to those reported in literature. Based on PIOPED criteria, large to medium sized pulmonary artery obstruction could be excluded in 25 of 26 sickle cell patients with PHT. As it was recently demonstrated that a low or intermediate probability VQ-scan virtually excludes medium to large pulmonary artery obstruction in idiopathic pulmonary hypertension, it is unlikely that significant pulmonary arterial obstruction was missed in these sickle cell patients. These data indicate that large to medium sized pulmonary artery obstruction (due to either pulmonary embolism or in situ thrombosis) is not important in the pathophysiology of SCD-related PHT in the majority of patients.

A detailed analysis of the VQ scans was performed in which all segments were separately studied in order to detect subtle perfusion abnormalities. Significantly more segments with irregular distribution of the radiopharmaceutical were detected in the left lower lobe of patients with PHT compared to patients without PHT. Given the anatomic localization this is likely explained by a more pronounced cardiomegaly. Neither general patchiness of perfusion, nor diffuse left to right lung perfusion differences, findings known to be associated with primary PHT, differed between sickle cell patients with and without PHT.

Previous studies have demonstrated a TRV≥2.5 m/s to be strongly indicative of PHT in SCD, and screening for SCD-related PHT with echocardiography is now generally recommended. In our cohort 27% of patients had only trace or no detectable TRV. Even though we cannot exclude that we have missed cases of PHT in this group, long term follow-up of sickle cell patients without a detectable TRV argues against a high prevalence of PHT in this group. Importantly, the VQ scintigraphy results in these patients were comparable to those in patients with a measurable TRV. As right heart catheterization remains the gold standard diagnostic test for PHT, we cannot exclude the possibility that some cases of PHT may have been missed or over-diagnosed. However, given the reported correlation between pulmonary artery pressure and the TRV in SCD, we feel that it is
unlikely that the lack of right heart catheterization in our patients would significantly alter our results.

Few studies have concentrated on VQ scintigraphy in SCD. Walker et al. demonstrated normal VQ scintigraphy results in 8 of 16 asymptomatic adult sickle cell patients, with 2 VQ-scans indicating pulmonary embolism. In a retrospective study, Kaur and colleagues demonstrated 3 intermediate probability VQ scans in 10 sickle cell patients presenting with chest pain. In a recent case control study in 26 sickle cell patients with and 17 without PHT, a high prevalence of patchy perfusion defects typical of idiopathic PHT was demonstrated in PHT patients, with 3 patients characterized by high probability VQ defects. These findings may be explained by the fact that these patients had more severe PHT (mean TRV 3.4m/s, range 2.8-4.2) as compared to the patients in our cohort (mean TRV 2.7m/s, range 2.5-3.5m/s with only three patients with a TRV > 2.8m/s). Therefore, in SCD, as in other forms of PHT, either acute or gradual obstruction of medium to large pulmonary arteries may well contribute to the progression of SCD-related PHT. However, our data do indicate that in sickle cell patients with relatively mild or early stage PHT, pulmonary artery obstruction is rare and therefore unlikely to be the an important causative or contributing factor in its primary etiology. Small pulmonary artery thrombosis or emboli, which are not detected with VQ scintigraphy, cannot be excluded. Based on the above, serial VQ scintigraphy may prove to be indicated in order to detect pulmonary artery obstruction as early as possible once PHT is diagnosed in patients with SCD. Whether intervention with anticoagulants will then retard the progression of PHT should be addressed in a randomized clinical trial.

In conclusion, our data demonstrate that large to medium sized pulmonary artery obstruction is not prevalent in ambulatory patients with SCD and is not associated with PHT in these patients. Pulmonary thrombo-emboli of large to medium sized pulmonary arteries are therefore unlikely to be a causative factor in SCD-related PHT.

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References


No association of the hypercoagulable state with sickle cell disease related pulmonary hypertension

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\textsuperscript{5}Department of Immunology, Red Cross Blood Bank Foundation, Curaçao, Netherlands Antilles.
\textsuperscript{6}Department of Hematology, Erasmus Medical Center, Rotterdam.
\textsuperscript{7}Department of Cardiology, Slotervaart Hospital, Amsterdam.
Abstract

Pulmonary hypertension (PHT) is a frequently occurring risk factor for early death in sickle cell disease (SCD). We set out to investigate whether the hypercoagulable state of SCD differs between sickle cell patients with and without PHT.

We compared thrombin generation, markers of thrombin generation (F1.2, TAT), soluble tissue factor antigen levels, markers of fibrinolysis (D-dimer, PAI-1), and platelet (sP-selectin) between consecutive sickle cell patients with (n=25) and without PHT (n=53). None of the measured parameters differed between patients with and without PHT. A modest but statistically significant relation between the haemolytic rate and the hypercoagulable state was detected.

In this study, the hypercoagulable state did not differ between sickle cell patients with and without PHT, arguing against a role of major importance in its pathophysiology.
Introduction

Pulmonary hypertension (PHT) occurs in approximately 30% of adult patients with sickle cell disease (SCD) and is an important risk factor for early death.\textsuperscript{1} The pathophysiology of SCD-related PHT has not been elucidated. In general, pulmonary artery thrombosis contributes to the progression of PHT regardless of its cause, forming the basis for instituting anticoagulation in patients with PHT.\textsuperscript{2} In SCD, several autopsy studies have shown a high prevalence of pulmonary artery thrombosis (albeit \textit{in situ} thrombosis or pulmonary embolism) and a recent study detected an increased prevalence of pulmonary perfusion abnormalities employing ventilation perfusion (VQ) scintigraphy in sickle cell patients with moderate to severe PHT.\textsuperscript{3,6} However, in a series of 78 consecutive adult sickle cell patients that underwent VQ scintigraphy, large to medium sized pulmonary artery obstruction was ruled out in 25 of 26 sickle cell patients with mostly mild PHT, indicating that large to medium sized pulmonary artery thromboembolism is probably a late event in SCD-related PHT and not of primary pathophysiological importance.\textsuperscript{7} However, organized thrombi in small pulmonary arteries, especially in association with obliterating vessel wall changes, do occur in SCD and may remain undetected with VQ scintigraphy.\textsuperscript{4,8,9} Sickle cell patients are characterized by a hypercoagulable state, with increased thrombin generation, enhanced fibrinolysis, and reduced levels and activity of naturally occurring anticoagulants.\textsuperscript{10} Moreover, a pro-thrombotic state has been linked to PHT in general as well. For example, patients with idiopathic and familial pulmonary hypertension are characterized by ongoing thrombin generation, platelet activation and increased levels of PAI-1 suggesting impaired fibrinolysis.\textsuperscript{10-12} The aim of the current study was to investigate whether the hypercoagulable state of SCD is associated with the occurrence of SCD-related PHT.
Design and methods

Citrated plasma samples were available to us from 78 of 85 adult sickle cell patients echocardiographically screened for PHT as reported elsewhere.\cite{1,7} Mild PHT is defined as a tricuspid regurgitant jet flow velocity (TRV) TRV of 2.5-2.9m/s, with moderate-severe PHT defined as TRV\geq3m/s. Pulmonary-artery pressures were considered normal in patients with trace or no tricuspid regurgitation (with the TRV assigned a value of 1.3 m/s in the latter group).\cite{1} The protocol was reviewed by a central medical ethical committee (Slotervaart Hospital) and subsequently reviewed and approved in the Academic Medical Center and Erasmus Medical Center. The study was carried out in accordance with the principles of the Declaration of Helsinki.

Collection of blood samples

Blood samples were taken from the antecubital vein with a vacutainer® system, the 3rd and 4th samples collected into 4.5mL tubes containing 0.105M buffered sodium citrate (Becton Dickinson, San Jose, CA). Citrated plasma was pooled and cells were removed by centrifugation for 15 minutes at 1550x G. (300RPM), and and stored at -80°C until analysis.

Endogenous thrombin potential (ETP)

The Calibrated Automated Thrombogram was obtained from Thrombinoscope BV, Maastricht, The Netherlands and all samples were analyzed according to the manufacturer’s instructions in a 96-well plate fluorometer (Ascent Reader, Thermolabsystems OY, Helsinki, Finland) equipped with a 390/460 filter set and a dispenser. After dilution addition of reagents and with platelet-poor plasma reagent, all wells contained 1 pM recombinant relipidated tissue factor (rTF) and 4 μM phosphatidylserine/ phosphatidylycholine/ phosphatidylethanolamine vesicles in HEPES-buffered saline. FluCa-solution contained HEPES, pH 7.35, 100 nM CaCl2, 60 mg/mL BSA, and 2.5 mM Z-Gly-Gly-Arg-AMC.

All plasma samples were analysed in duplicate and each measurement comprised a thrombin generation and calibrator analysis. In brief, 80 μl of plasma was added to each well and thrombin generation wells received 20 μl of platelet-poor plasma reagent, and the calibrator wells received 20 μl calibrator containing α2 macroglobulin-thrombin complex. Microtiter plates were placed in the fluorometer and incubated at 37°C for 5 minutes. The dispenser of the fluorometer was flushed with a warm 100 mM CaCl2 solution (37°C) and subsequently flushed with FluCa. Thrombin generation was started by addition of 20 μl FluCa to each well and recorded by means of the Thrombinoscope software. Five parameters were derived from the thrombin generation curve: lag time, endogenous thrombin potential (ETP), peak height, time to maximum peak height, and time to end of peak. Thrombin generation was also performed with the addition of thrombomodulin (a
kind gift from Prof. C. Hemker, Synapse BV, Maastricht.) at a concentration that upon addition to
normal pool plasma would reduce the ETP value with 50%.

Assays
The following haemostatic markers were determined according to manufacturer’s procedures;
prothrombin fragment 1+2 (F1+2), soluble tissue factor and P-selectin (ELISA; Behring, Marburg,
Germany), D-dimers (TintElize D-Dimer assay, Trinity Biotech, Kordia Life Sciences),
plasminogen activator inhibitor-1 activity (PAI-1:act, Chromolize, Trinity Biotech).

Statistics
For data analysis, the more severe disease genotypes HbSS and HbSβ0-thalassemia were grouped
together, as were the relatively milder disease genotypes HbSβ+ -thalassemia and HbSC. All
numbers are medians with corresponding inter-quartile range (IQR). Between group differences
were tested with the Mann-Whitney U test. For correlation studies the Spearman rank correlation
coefficient (r_s) was calculated. P-values <0.05 were considered statistically significant (SPSS 12.0.2,
SPSS Inc, Chicago, IL).
Results and discussion

Patient characteristics and results of measured parameters are shown in Table 1. Our PHT patients consisted almost exclusively of patients with mild PHT (with only two patients with a TRV ≥3.0 m/s). As only three patients in the HbSC/HbSβ+-thalassemia group had PHT no statistical analysis pertaining to between group differences was performed in these patients. Use of hydroxyurea was not different between patients with or without PHT. None of the patients were on anticoagulation or used calcium antagonists, endothelin receptor blockers or sildenafil.

<table>
<thead>
<tr>
<th>Table 1. Results echocardiography and coagulation studies.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td><strong>HbSS/HbSβ⁺-thalassemia</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Non-PHT</strong> (n=32)</td>
</tr>
<tr>
<td><strong>PHT</strong> (n=22)</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>33 (21-46)</td>
</tr>
<tr>
<td>30 (24-52)</td>
</tr>
<tr>
<td>Male-female</td>
</tr>
<tr>
<td>6:26</td>
</tr>
<tr>
<td>6:16</td>
</tr>
<tr>
<td>Haemoglobin (mmol/L)</td>
</tr>
<tr>
<td>5.6 (4.9-6.0)</td>
</tr>
<tr>
<td>5.0 (4.3-5.9)</td>
</tr>
<tr>
<td>Fetal haemoglobin (%)</td>
</tr>
<tr>
<td>9.8 (5.6-17.6)</td>
</tr>
<tr>
<td>6.9 (2.2-13.4)</td>
</tr>
<tr>
<td>LDH (U/L)</td>
</tr>
<tr>
<td>389 (323-526)</td>
</tr>
<tr>
<td>543 (370-735)</td>
</tr>
<tr>
<td>TRV (m/s)</td>
</tr>
<tr>
<td>1.9 (1.3-2.3)*</td>
</tr>
<tr>
<td>2.6 (2.6-2.8)*</td>
</tr>
<tr>
<td>Coagulation activation:</td>
</tr>
<tr>
<td>TAT (µg/l)</td>
</tr>
<tr>
<td>4.7 (3.7-6.1)</td>
</tr>
<tr>
<td>3.9 (2.9-5.9)</td>
</tr>
<tr>
<td>Fvwt (pmol/l)</td>
</tr>
<tr>
<td>305 (245-368)</td>
</tr>
<tr>
<td>267 (173-401)</td>
</tr>
<tr>
<td>PAI-1 (IU/ml)</td>
</tr>
<tr>
<td>1.8 (0.9-2.2)</td>
</tr>
<tr>
<td>1.7 (0.9-2.7)</td>
</tr>
<tr>
<td>D-dimer (µg/l)</td>
</tr>
<tr>
<td>288 (248-373)</td>
</tr>
<tr>
<td>250 (162-475)</td>
</tr>
<tr>
<td>Tissue factor:</td>
</tr>
<tr>
<td>sTF (pg/ml)</td>
</tr>
<tr>
<td>177 (158-240)</td>
</tr>
<tr>
<td>221 (174-286)</td>
</tr>
<tr>
<td>Platelet count and activation:</td>
</tr>
<tr>
<td>sP-selectin (ng/ml)</td>
</tr>
<tr>
<td>33 (27-47)</td>
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<tr>
<td>37 (28-46)</td>
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<tr>
<td>Platelet count (x10⁹/L)</td>
</tr>
<tr>
<td>308 (246-377)</td>
</tr>
<tr>
<td>298 (217-382)</td>
</tr>
<tr>
<td>Thrombin generation:</td>
</tr>
<tr>
<td>ETP (nM-min)</td>
</tr>
<tr>
<td>1082 (920-1237)</td>
</tr>
<tr>
<td>1062 (935-1124)</td>
</tr>
<tr>
<td>1147 (1089-1393)</td>
</tr>
<tr>
<td>1315 (1052-1578)</td>
</tr>
<tr>
<td>ETP-TM (nM-min)</td>
</tr>
<tr>
<td>1054 (869-1173)</td>
</tr>
<tr>
<td>1012 (895-1086)</td>
</tr>
<tr>
<td>1076 (997-1318)</td>
</tr>
<tr>
<td>1267 (995-1538)</td>
</tr>
<tr>
<td>% change adding TM</td>
</tr>
<tr>
<td>3.1 (2.3-6.9)</td>
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<td>4.6 (3.3-6.3)</td>
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<tr>
<td>6.7 (3.4-9.2)</td>
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<tr>
<td>4.0 (2.5-5.4)</td>
</tr>
<tr>
<td>ETP-lipid (nM-min)</td>
</tr>
<tr>
<td>1030 (917-1151)</td>
</tr>
<tr>
<td>1017 (887-1072)</td>
</tr>
<tr>
<td>1132 (1049-1336)</td>
</tr>
<tr>
<td>1290.5 (989-1592)</td>
</tr>
<tr>
<td>% change without lipid</td>
</tr>
<tr>
<td>3.7 (1.8-6.7)</td>
</tr>
<tr>
<td>5.6 (2.7-7.4)</td>
</tr>
<tr>
<td>2.9 (1.3-4.9)</td>
</tr>
<tr>
<td>2.6 (-0.9-6.0)</td>
</tr>
</tbody>
</table>

*Statistically significant (P<0.05) between PHT and Non-PHT. PHT = pulmonary hypertension. LDH = Lactate Dehydrogenase. ETP = Endogenous Thrombin Potential with 1 pmol TF and 4 µmol lipid (AUC in nM-min). ETP-TM = ETP with addition of thrombomodulin. ETP-lipid = ETP without addition of lipids.
No differences could be detected between any of the measured parameters between sickle cell patients with and without PHT. Furthermore, there was no statistically significant correlation of any measured parameter to the TRV values. Recently, a study in patients with β-thalassemia, a chronic form of haemolytic anemia also characterized by a high incidence of PHT, also failed to detect a difference in hypercoagulable state or endothelial activation between patients with and without PHT, except for higher plasma P-selectin levels and platelet counts in patients with PHT (the latter may be explained by a higher number of PHT patients with a history of a splenectomy).13 Based upon our data, a role of major importance in the early pathophysiology of SCD-related PHT seems relatively unlikely even though a contributing role of the hypercoagulable state in advanced SCD-related PHT cannot be ruled out.

An elegant study demonstrated that the hypercoagulable state is closely linked to the rate of red cell phosphatidylserine (PS) exposure as result the repetitive sickling and unsickling cycles, with both higher PS expression and coagulation activation in HbSS patients as opposed to HbSC patients.16 In line with these findings, a modest but statistically significant correlation in especially HbSS/HbSβ0-thalassemia patients of the rate of haemolysis to plasma concentrations of haemostatic markers was detected (see table 2). The inverse relation of the in vitro thrombin generation markers of haemolysis is therefore likely due to a reduced availability of coagulation factors in patients with more outspoken haemolysis (and hence higher PS expression).17 The demonstration of the marked in vitro resistance to thrombomodulin indicates functional protein C resistance, which is in accordance with previously reported reduced protein C and S levels in SCD.18

<p>| Table 2. Correlation studies between markers of hemolysis and the hypercoagulable state. |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th>F1+2 (µmol/L)</th>
<th>Hb</th>
<th>LDH</th>
<th>Ret</th>
<th>Hb</th>
<th>LDH</th>
<th>Ret</th>
<th>Hb</th>
<th>LDH</th>
<th>Ret</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.26</td>
<td>0.40</td>
<td>0.38</td>
<td>-0.13</td>
<td>0.33</td>
<td>0.26</td>
<td>-0.02</td>
<td>0.18</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>(0.02)</td>
<td>(&lt;0.01)</td>
<td>(&lt;0.01)</td>
<td>(0.35)</td>
<td>(0.02)</td>
<td>(0.08)</td>
<td>(0.94)</td>
<td>(0.40)</td>
<td>(0.01)</td>
<td></td>
</tr>
<tr>
<td>TAT (µmol/L)</td>
<td>-0.35</td>
<td>0.44</td>
<td>0.41</td>
<td>-0.26</td>
<td>0.35</td>
<td>0.33</td>
<td>-0.10</td>
<td>0.33</td>
<td>0.30</td>
</tr>
<tr>
<td>(&lt;0.01)</td>
<td>(&lt;0.01)</td>
<td>(&lt;0.01)</td>
<td>(0.06)</td>
<td>(0.01)</td>
<td>(0.02)</td>
<td>(0.66)</td>
<td>(0.13)</td>
<td>(0.17)</td>
<td></td>
</tr>
<tr>
<td>D-dimer (µg/l)</td>
<td>-0.36</td>
<td>0.41</td>
<td>0.43</td>
<td>-0.33</td>
<td>0.41</td>
<td>0.45</td>
<td>-0.24</td>
<td>0.11</td>
<td>0.26</td>
</tr>
<tr>
<td>(&lt;0.01)</td>
<td>(&lt;0.01)</td>
<td>(&lt;0.01)</td>
<td>(0.02)</td>
<td>(&lt;0.01)</td>
<td>(&lt;0.01)</td>
<td>(0.27)</td>
<td>(0.62)</td>
<td>(0.23)</td>
<td></td>
</tr>
<tr>
<td>ETP (nM-min)</td>
<td>0.28</td>
<td>-0.28</td>
<td>-0.22</td>
<td>0.15</td>
<td>-0.17</td>
<td>-0.04</td>
<td>0.28</td>
<td>-0.13</td>
<td>-0.24</td>
</tr>
<tr>
<td>(0.01)</td>
<td>(0.01)</td>
<td>(0.06)</td>
<td>(0.28)</td>
<td>(0.21)</td>
<td>(0.76)</td>
<td>(0.19)</td>
<td>(0.53)</td>
<td>(0.27)</td>
<td></td>
</tr>
<tr>
<td>ETP-lipid (nM-min)</td>
<td>0.34</td>
<td>-0.33</td>
<td>-0.30</td>
<td>-0.2</td>
<td>-0.2</td>
<td>-0.10</td>
<td>0.44</td>
<td>-0.19</td>
<td>-0.35</td>
</tr>
<tr>
<td>(&lt;0.01)</td>
<td>(&lt;0.01)</td>
<td>(&lt;0.01)</td>
<td>(0.15)</td>
<td>(0.15)</td>
<td>(0.52)</td>
<td>(0.04)</td>
<td>(0.38)</td>
<td>(0.10)</td>
<td></td>
</tr>
<tr>
<td>ETP-TM (nM-min)</td>
<td>0.21</td>
<td>-0.20</td>
<td>-0.15</td>
<td>-0.11</td>
<td>-0.11</td>
<td>0.02</td>
<td>0.38</td>
<td>-0.11</td>
<td>-0.31</td>
</tr>
<tr>
<td>(0.07)</td>
<td>(0.08)</td>
<td>(0.21)</td>
<td>(0.42)</td>
<td>(0.42)</td>
<td>(0.87)</td>
<td>(0.08)</td>
<td>(0.63)</td>
<td>(0.15)</td>
<td></td>
</tr>
</tbody>
</table>

Data are shown as Spearman rank correlation coefficients with corresponding P-values between brackets. Statistically significant correlations are depicted in bold. Hb=haemoglobin (mmol/L), LDH=lactate dehydrogenase(U/L), Ret=reticulocytes (%).
In interpreting these data several shortcomings need to be taken into account. Importantly, our findings only pertain to HbSS and HbSβ-thalassemia patients with mild PHT. Secondly, right heart catheterization remains the gold standard diagnostic test for PHT and is recommended in sickle cell patients with moderate-severe PHT. This invasive procedure has not been carried out in these patients. We therefore cannot exclude the possibility that some cases of PHT may have been missed or over-diagnosed. However, given the reported correlation between pulmonary artery pressure and the TRV in SCD, and given the fact an elevated TRV is solely the result of left-sided heart disease in a minority of cases, we do not feel that the lack of right heart catheterization in our patients would significantly alter our results. In conclusion, we could not demonstrate a difference in the hypercoagulable state in sickle cell patients with or without PHT. The hypercoagulable state seems to be related to the rate of haemolysis, and haemolysis has been recognized as a risk factor for developing PHT not only in SCD but in other forms of chronic haemolysis as well. However, given the lack of an association of the hypercoagulable state to SCD-related PHT, other untoward effects of haemolysis, such as nitric oxide scavenging, therefore seem of greater primary importance in its pathophysiology.
References


Association of asymmetric dimethylarginine with sickle cell disease related pulmonary hypertension

(Based on: Haematologica. 2008 Jul 18. [Epub ahead of print])

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³Department of Hematology, Academic Medical Center, Amsterdam.
⁴Department of Internal Medicine, Slotervaart Hospital, Amsterdam.
⁵Department of Pathology and Laboratory Medicine, University Hospital Groningen, Groningen.
⁶Department of Haematology, Erasmus Medical Center, Rotterdam.
⁷Department of Cardiology, Slotervaart Hospital, Amsterdam.
Abstract

Reduced nitric oxide (NO) bioavailability plays a major role in the development of sickle cell disease (SCD) related pulmonary hypertension (PHT). We investigated whether asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase inhibitor, could play a role in SCD-related PHT.

Plasma ADMA and serum sVCAM-1 concentrations were determined in adult sickle cell patients consecutively screened for PHT with echocardiography.

Plasma ADMA concentrations were increased in HbSS/HbSβ⁰-thalassemia patients with PHT (n=18, median 0.63 μmol/mL, inter quartile range 0.58-0.79) as compared to those without PHT (n=28, 0.57 μmol/mL, 0.52-0.65) (p=0.01) and significantly correlated to the haemolytic rate. Also, ADMA and sVCAM-1 were significantly correlated to the tricuspid regurgitant jet flow velocity (rₐ=0.33, p=0.03 and rₐ=0.49, p=0.002, respectively), and to each other (rₐ=0.59, p<0.001).

ADMA may be a contributing factor to the development of SCD related PHT also linking haemolysis and endothelial activation.
Introduction

Pulmonary hypertension (PHT) occurs in approximately 30% of adult sickle cell patients and is associated with a high risk of early death. Reduced nitric oxide (NO) bioavailability secondary to chronic intra-vascular haemolysis is considered to be of primary importance in the pathogenesis of sickle cell disease (SCD) related PHT. Chronic NO shortage leads to pulmonary artery vasoconstriction, endothelial activation and vascular remodeling resulting in or contributing to an obliterative vasculopathy.

Recently we reported elevated plasma concentrations of asymmetric dimethylarginine (ADMA) in SCD, revealing another contributing factor in limiting NO bioavailability. ADMA, as well as \( N^\epsilon,N^\epsilon \)-dimethyl-L-arginine (symmetric dimethylarginine or SDMA), derive from the irreversible post-translational methylation of arginine residues by protein arginine methyltransferases (PMRT) and are released as free amino acids upon proteolysis. ADMA (but not SDMA) competitively inhibits the NO synthase (NOS) enzyme system, thereby limiting NO production. ADMA is degraded by dimethylarginine dimethylaminohydrolases (DDAH) whereas SDMA is mainly cleared renally. Elevated plasma ADMA concentrations have been reported in several forms of PHT and have been linked to outcome in PHT as well. The aim of the current study was to investigate whether ADMA concentrations are associated with PHT in SCD.
Patients and methods

Patients
Serum and EDTA plasma samples were available to us from adult sickle cell patients consecutively screened for PHT with echocardiography as reported elsewhere.9 Mild and moderate-severe PHT are defined as a tricuspid regurgitant jet flow velocity (TRV) of 2.5-2.9m/s and TRV≥3m/s respectively, pulmonary-artery pressures are considered normal in patients with trace or no tricuspid regurgitation (with TRV assigned 1.3 m/s).1 The study protocol was reviewed by a central medical ethical committee (Slotervaart Hospital) and subsequently reviewed and approved in the Academic Medical Center and Erasmus Medical Center. All patients gave written and informed consent. The study was carried out in accordance with the principles of the Declaration of Helsinki.

Laboratory determinations
Plasma concentrations of arginine, ADMA, and SDMA were measured by high-performance liquid chromatography with fluorescence detection as previously described,10 with modified chromatographic separation conditions.11 The inter-assay coefficient of variation was <3% for all compounds. Plasma amino acids were determined by ion-exchange chromatography with ninhydrin postcolumn derivatization and colorimetric detection. Serum soluble vascular cell adhesion molecule-1 (sVCAM-1) levels were determined according to manufacturer’s procedures (R&D Systems USA).

Statistics
Between group differences were tested with the Mann-Whitney U test. For correlation studies the Spearman rank correlation coefficient (r_s) was calculated. P-values <0.05 were considered statistically significant (SPSS 12.0.2, SPSS Inc, Chicago, IL).
Results

Patients

Only three of 24 HbSC/HbSβ+-thalassemia patients had PHT, therefore only data regarding HbSS/HbSβ+-thalassemia patients are reported (see Table 1). Our PHT patients consisted almost exclusively of patients with mild PHT (only two patients with a TRV≥3.0m/s). Use of hydroxyurea was not different between patients with and without PHT and no patients used anticoagulation or calcium antagonists, endothelin receptor blockers or sildenafil.

<p>| Table 1. Demographics and laboratory parameters in sickle cell patients with and without PHT. |
|---------------------------------------------------------------|---------------------------------|----------------|-----|</p>
<table>
<thead>
<tr>
<th>N</th>
<th>without PHT</th>
<th>PHT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>28</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>33 (21-44)</td>
<td>28 (22-52)</td>
<td>0.80</td>
</tr>
<tr>
<td>Male:female</td>
<td>6:22</td>
<td>5:13</td>
<td></td>
</tr>
<tr>
<td>TRV (m/s)</td>
<td>2.0 (1.3-2.3)</td>
<td>2.7 (2.6-2.8)</td>
<td></td>
</tr>
<tr>
<td>sPAP* (mmHg)</td>
<td>21 (12-28)</td>
<td>34 (32-43)</td>
<td></td>
</tr>
<tr>
<td>Hb (mmol/L)</td>
<td>5.7 (5.0-6.2)</td>
<td>4.9 (4.2-5.9)</td>
<td>0.05</td>
</tr>
<tr>
<td>HbF (%)</td>
<td>10.6 (6.1-18.3)</td>
<td>5.9 (2.2-14.1)</td>
<td>0.13</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>369 (300-515)</td>
<td>575 (388-846)</td>
<td>0.02</td>
</tr>
<tr>
<td>GFR** (mL/min)</td>
<td>151 (120-195)</td>
<td>120 (66-172)</td>
<td>0.10</td>
</tr>
<tr>
<td>ADMA (µmol/L)</td>
<td>0.57 (0.52-0.65)</td>
<td>0.63 (0.58-0.79)</td>
<td>0.01</td>
</tr>
<tr>
<td>SDMA (µmol/L)</td>
<td>0.47 (0.42-0.55)</td>
<td>0.51 (0.47-0.83)</td>
<td>0.07</td>
</tr>
<tr>
<td>Arginine (µmol/L)</td>
<td>45 (32-56)</td>
<td>46 (41-62)</td>
<td>0.26</td>
</tr>
<tr>
<td>Ornithine (µmol/L)</td>
<td>56 (42-66)</td>
<td>56 (45-75)</td>
<td>0.41</td>
</tr>
<tr>
<td>Citrulline (µmol/L)</td>
<td>23 (16-32)</td>
<td>27 (20-32)</td>
<td>0.66</td>
</tr>
<tr>
<td>Proline (µmol/L)</td>
<td>208 (162-257)</td>
<td>209 (176-234)</td>
<td>0.84</td>
</tr>
<tr>
<td>Arginine/ornithine</td>
<td>0.84 (0.66-1.0)</td>
<td>0.93 (0.72-1.15)</td>
<td>0.45</td>
</tr>
<tr>
<td>Arginine/citrulline</td>
<td>1.87 (1.66-2.49)</td>
<td>1.98 (1.43-2.65)</td>
<td>0.84</td>
</tr>
<tr>
<td>Arginine/proline</td>
<td>0.23 (0.18-0.31)</td>
<td>0.25 (0.19-0.35)</td>
<td>0.41</td>
</tr>
<tr>
<td>sVCAM-1 (ng/mL)</td>
<td>1089 (801-1239)</td>
<td>1542 (1119-1880)</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Data are presented as medians with their corresponding inter quartile range. A p-value <0.05 is considered statistically significant. *Right ventricular systolic pressure was estimated based on the modified Bernoulli equation (I) and considered to be equal to the systolic pulmonary artery pressure (sPAP) in absence of right ventricular outflow obstruction. **Cockcroft and Gault-formula (males: creatinine clearance= 1.23 x weight x (140-age) / serum creatinine, females: creatinine clearance = 1.03 x weight x (140-age) / serum creatinine).
ADMA, SDMA, amino-acid profiles and endothelial activation

Plasma ADMA concentrations were significantly increased in HbSS/HbSβ+-thalassemia patients with PHT as compared to those without PHT. There were no differences in concentrations of SDMA, arginine, ornithine, citrulline and proline and their ratios between patients with and without PHT. Serum sVCAM-1 levels were significantly higher in patients with PHT (Table 1).

Both plasma ADMA and serum sVCAM-1 concentrations were significantly correlated to the TRV ($r_s=0.33$, $p=0.03$ and $r_s=0.49$, $p=0.002$, respectively), with a significant correlation between ADMA and sVCAM-1 as well ($r_s=0.59$, $p<0.001$). Statistically significant correlations of ADMA with haemoglobin concentrations ($r_s=-0.47$, $p=0.001$), HbF% ($r_s=-0.50$, $p=0.01$) and LDH levels ($r_s=0.54$, $p<0.001$) were observed. As expected, SDMA, but not ADMA, concentrations were significantly correlated to the glomerular filtration rate (GFR) ($r_s=-0.66$, $p<0.001$, $r_s=-0.08$, $p=0.60$, respectively). SDMA was also significantly correlated to ADMA concentrations, haemoglobin concentrations ($r_s=-0.51$, $p=0.001$), and LDH ($r_s=0.31$, $p=0.04$). Haemoglobin concentrations were significantly correlated to TRV ($r_s=-0.30$, $p=0.04$). There was no statistically significant correlation between age and TRV, ADMA concentrations, haemoglobin concentrations, LDH levels and sVCAM-1 levels (data not shown), whereas both GFR en SDMA were significantly correlated to age ($r_s=-0.58$, $p<0.001$, $r_s=0.52$, $p<0.001$, respectively).
Discussion

Haemolysis driven reductions in NO bioavailability as a result of both NO scavenging by cell free haemoglobin and increased arginase activity are of major importance in the pathophysiology of SCD related PHT.\(^2\) Over the last years ADMA has been recognized as an important regulator of NO production by inhibiting NOS activity.\(^6\) and plasma ADMA concentrations are elevated in SCD.\(^{4,5}\) In this study we demonstrate an association of plasma ADMA concentrations with SCD related PHT, haemolysis and endothelial activation.

Plasma ADMA concentrations were higher in patients with PHT as opposed to patients without PHT, with a modest but statistically significant correlation between the TRV and plasma ADMA concentrations. ADMA concentrations in patients without PHT were elevated as compared to values previously reported by us in healthy race matched controls.\(^{4,5}\) Clearly, patients with the highest haemolytic rate are characterized by highest plasma ADMA and SDMA concentrations indicating that the haemolytic rate may be an important determinant of methylarginine production in SCD (likely due to the increased protein turn-over in the stress erythropoiesis). Specific factors related to the pulmonary vasculature contributing to ADMA increments in SCD could be shear stress induced PMRT activity and hypoxia induced DDAH down-regulation.\(^{12-14}\) Although the ADMA difference between patients with and without PHT seems modest, even small extra-cellular ADMA increments lead to significant intra-cellular NOS inhibition through preferred cellular uptake of ADMA over arginine.\(^6\) A recent study demonstrated a clear association of plasma ADMA concentrations ≥0.64μmol/L with strongly reduced pulmonary artery endothelial NOS expression and early death in patients with thrombo-embolic PHT.\(^8\)

ADMA induced NOS inhibition is associated with endothelial activation and dysfunction.\(^{15,16}\) In our study serum sVCAM-1 levels strongly correlated to plasma ADMA concentrations, indicating that ADMA induced NOS inhibition may be an important contributor to the characteristic endothelial activation of SCD.\(^{17}\) As reported by others, serum sVCAM-1 levels correlated significantly to the TRV in our patients.\(^{18}\) Given the strong relation of ADMA to endothelial activation, it would be interesting to hypothesize that chronic haemolysis induced ADMA elevations significantly contribute to endothelial activation and dysfunction via NOS inhibition in SCD and that patients with higher ADMA concentrations are more prone to develop a vasculopathy leading to complications such as PHT over time.

Previous studies have demonstrated an association of increased arginase activity (reflected by lower arginine to ornithine ratios) with severe, but not mild SCD related PHT.\(^{1,19}\) In accordance with the studies above, no difference in arginine/ornithine ratios between our patients with mostly mild PHT and patients without PHT could be detected, indicating similar arginase activity. The
increased plasma ADMA concentrations in mild SCD related PHT therefore could suggest a role of pathophysiological importance at an earlier stage than increased arginase activity. In interpreting these data the relatively small number of patients needs to be taken into account and these findings need to be reproduced in a larger cohort. Also, right heart catheterization remains the gold standard diagnostic test for PHT and is recommended in sickle cell patients with moderate-severe PHT. However, given the reported excellent correlation between pulmonary artery pressure and TRV in SCD, and the fact that an elevated TRV is the result of solely left-sided heart disease in only a minority of cases, we do not feel that the lack of right heart catheterization would significantly affect our results. Taken together, our data identify a potential role of ADMA as a novel early contributing factor to the development of PHT in SCD. Also, ADMA induced limitation of NO production may well provide an important new mechanistic link between haemolysis and the characteristic endothelial activation of SCD.

Acknowledgment

We gratefully acknowledge the expert technical assistance of Sigrid de Jong.
References


Chapter 9


Cardiopulmonary imaging, functional and laboratory studies in sickle cell disease associated pulmonary hypertension

(Accepted: Am J Hematol)

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Abstract

Pulmonary hypertension (PHT) occurs in approximately 30% of adults with sickle cell disease (SCD) and is an independent risk factor for early death. In this study, we aimed to determine the value of general laboratory testing, plain chest radiography, electrocardiography (ECG), high resolution computer tomography (HRCT) of the thorax, pulmonary function testing and plasma N-terminal brain natriuretic peptide (NT-proBNP) and brain natriuretic peptide (BNP) in patients with SCD-related PHT.

A cohort of 85 ambulatory sickle cell patients were prospectively screened for PHT with echocardiography (defined as a tricuspid regurgitation flow velocity (TRV) of ≥2.5 m/s). All patients were systematically evaluated by the aforementioned diagnostic tests comparing patients with and without PHT.

The prevalence of PHT was 41% in HbSS/HbSβ0-thalassemia patients and 13% in HbSC/HbSβ+ -thalassemia patients. No statistically significant differences were detected in ECG, chest radiography, HRCT and pulmonary function testing between patients with and without PHT. The degree of anemia and renal dysfunction, but not the presence of PHT, were the most important determinants of plasma (NT-pro)BNP levels.

The performed imaging and functional studies do not seem to be of value in identifying etiological conditions (such as airflow obstruction or parenchymal lung disease), nor do they offer clues to the presence of mild PHT in SCD.
Introduction

Patients with sickle cell disease (SCD) are characterized by recurrent microvascular occlusion, chronic hemolytic anemia and an increased susceptibility to infections. Due to the advent of efficacious therapeutic modalities for treatment and prevention of acute SCD related complications the mean life expectancy for this group of patients is on the rise in the Western World. In order to further improve outcome in patients with SCD, early recognition and management of chronic disease related complications is becoming a primary concern. Recently, pulmonary hypertension (PHT) has been recognized as a severe SCD related complication. It occurs in approximately 30% of adult patients and is an independent risk factor for early death.1-3 Due to the non specific clinical manifestations (or absence of manifestations) of PHT in SCD, non-invasive screening for PHT employing trans-thoracic echocardiography is nowadays recommended. In daily clinical practice, once a patient is diagnosed with PHT, often an array of tests is performed in order to identify possible causes and/or aggravating factors. Examples are pulmonary imaging studies to detect parenchymal lung disease, pulmonary function testing in order to rule out air-flow obstruction and/or restrictive lung disease, electrocardiography to detect arrhythmia. Also, the presence of systolic and diastolic dysfunction is assessed with echocardiography and base-line electrocardiography should be performed. So far only a few studies reported on the value of employing such diagnostic tests is in sickle cell patients in relation to PHT.2-4 We report findings of such diagnostic tests performed in a cohort of sickle cell patients consecutively screened for PHT with echocardiography.
Methods

Patients
Consecutive adult sickle cell patients visiting the outpatient hematology/ internal medicine clinics of the Academic Medical Center (AMC, Amsterdam, the Netherlands), the Slotervaart Hospital (Amsterdam, the Netherlands) and the Erasmus Medical Center (Rotterdam, the Netherlands) were eligible for this study. Since the recognition of the high incidence of PHT in SCD and the associated high mortality we screened our all our patients for PHT employing echocardiography. In the first two years, we screened for generally known risk factors for PHT (parenchymal lung disease, lung function abnormalities) as well. Written informed consent was obtained from all patients for anonymously using all acquired clinical and laboratory data pertaining to their cardiopulmonary status for purpose of this analysis. Inclusion criteria were: high performance liquid chromatography (HPLC) confirmed diagnosis of homozygous sickle cell anemia (HbSS), sickle-C disease (HbSC), HbS\(\beta^+\)-thalassemia or HbS\(\beta^0\)-thalassemia, age 18 years or older and written informed consent. Exclusion criteria were: known congestive heart failure, history of chronic obstructive pulmonary disease (COPD) or poorly controlled asthma, a painful crisis in the preceding four weeks and/or acute chest syndrome in the preceding three months before performing any of the tests reported. The protocol was reviewed by the central medical ethical committee of the Slotervaart Hospital and subsequently reviewed and approved in the Academic Medical Center and Erasmus Medical Center. The study was carried out in accordance with the principles of the Declaration of Helsinki.

Trans-thoracic echocardiography
Trans-thoracic echocardiography was performed as described elsewhere. Mild PHT was defined as a tricuspid regurgitant jet flow velocity (TRV) of 2.5–2.9 m/s, with a TRV ≥3 m/s indicating moderate-severe PHT (referentie). Pulmonary artery pressure was considered normal in patients with trace or no tricuspid regurgitation (with the TRV assigned a value of 1.3 m/s in the latter group). An early (E) to atrial (A) ventricular filling ratio <1.0 indicated diastolic dysfunction.
Laboratory testing
Blood was drawn via venipuncture from the antecubital vein after patients were at rest in the supine position for twenty minutes. Standard hematological and biochemical parameters were directly measured according to local protocols. The percentage of fetal hemoglobin (HbF) was measured with high performance liquid chromatography. Remaining blood was centrifuged at 3,000 rpm for 15 min directly after collection in order to prepare EDTA plasma, citrated plasma and serum samples that were subsequently stored at -80°C until further analysis. Quantitative BNP and NT-proBNP levels were measured in EDTA-plasma with a microparticle enzyme immunoassay (Abbott Diagnostics) and an electrochemiluminescence immunoassay (Roche Diagnostics), respectively.

Chest x-rays
Standard anterior-posterior chest x-rays were performed in the erect position. Cardiotoracic ratio was calculated by dividing the transverse cardiac diameter by transverse diameter of the thorax on a standing postero-anterior (PA) chest radiograph, taken at a standard 2m patient-film distance and at deep inspiration. The transverse cardiac diameter was the sum of the maximum extensions of the heart to the right and left of the midline. The thoracic diameter was measured as the horizontal distance between the inner borders of the ribs at the level of the right diaphragm.6

High Resolution Computerized tomography
All HRCT scans were obtained in the supine position, at full inspiration, with 1.0-1.5 mm collimation, either at 10 mm intervals or as continuous volume spiral CT scan. Images were transferred in a central picture archiving and communication system (IMPAX) for clinical interpretation. All HRCT images were reviewed centrally by an experienced radiologist blinded to clinical data. HRCT scores were determined using a modification (see supplemental data) of a previously described scoring system for SCD.3 In addition, the ratio of artery to bronchus diameter was estimated in each lung quadrant for the hilar, medial and peripheral region. An artery to bronchus ratio ≥ 1 has previously been reported as an indicator of PHT.17
Pulmonary function testing
Forced expiratory volume in one second (FEV₁), forced vital capacity (FVC) and the ratio between FEV₁ and FVC (FEV₁/FVC) were measured using a pneumotachograph. Lung volumes were assessed by measurement of total lung capacity (TLC) using a constant volume whole-body plethysmography. Total lung gas transfer for carbon monoxide (T_{LCO} SB), alveolar volume (VA) and gas transfer per unit alveolar volume of carbon monoxide (T_{LCO} SB VA) were assessed using the single-breath gas transfer technique. Steady-state hemoglobin levels were measured during routine outpatient visits within 1 month of testing and were used to correct T_{LCO} and T_{LCO} VA values for hemoglobin level according to ATS/ERS criteria.¹⁸ Both spirometry, plethysmography and T_{LCO} were expressed as a percentage of predicted values according to the ATS/ERS standard.¹⁸-²⁰ A restrictive ventilatory defect is defined by a reduction in TLC below the 5th percentile of the predicted value with a normal FEV₁/VC. An obstructive ventilatory defect is defined by a reduced FEV₁/VC ratio below the 5th percentile of the predicted value.

Electrocardiography
Standard twelve-lead electrocardiograms (paper speed, 25 mm/s; sensitivity of 1 mV/10 mm) were performed in the supine position by certified ECG technicians. The electrocardiographic parameters evaluated included rate and rhythm, PR, PQ and QRS duration, mean frontal plane axis of the P, QRS and T wave. The following signs of right ventricular hypertrophy were assessed: right axis deviation of +110° or more, a dominant R wave in lead V1 or a R wave in lead V1 larger than 7 mm and P-pulmonale, defined as a tall P wave (height >2.5 mm) in leads II, III, and aVF.²¹ Electrocardiographic variables were evaluated by two investigators blinded to clinical and hemodynamic data and outcome. Any discrepancies between investigators were resolved by joint review.
Statistics
All numbers are medians (with corresponding inter quartile ranges) unless stated otherwise. Difference in continuous data between groups was tested with the Mann-Whitney U test. Difference in categorical data between groups was tested with the Pearson Chi-square test or the Fisher’s exact test when appropriate. Bivariate correlations were assessed with the spearman rank correlation coefficient (rₚ). Multi-variant analysis was assessed with linear regression models. A P-value < 0.05 was considered statistically significant. Missing data were regarded as missing at random. Statistical analysis was performed by using SPSS 12.0.2 (SPSS Inc, Chicago, IL).
Results

Patients
In total, 90 consecutive patients were eligible for this study of which 85 gave written informed consent.

For data analysis, the more severe SCD genotypes, HbSS and HbSβ0-thalassemia, were grouped together, as were the relatively milder SCD genotypes HbSβ+-thalassemia and HbSC. For patient characteristics and laboratory data see Table 1. Use of hydroxyurea was not different between patients with or without PHT (Table 1). Patients on hydroxyurea had comparable hemoglobin concentrations and serum lactate dehydrogenase levels but a higher HbF% as compared to patients not on hydroxyurea (data not shown).

<table>
<thead>
<tr>
<th>Table 1. Patient characteristics.</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td>Demographics:</td>
</tr>
<tr>
<td>Number of patients</td>
</tr>
<tr>
<td>Age (y)</td>
</tr>
<tr>
<td>Male/Female ratio</td>
</tr>
<tr>
<td>History of ACS (%)</td>
</tr>
<tr>
<td>On Hydroxyurea (%)</td>
</tr>
<tr>
<td>Echocardiography parameters:</td>
</tr>
<tr>
<td>TRV (m/s)</td>
</tr>
<tr>
<td>sPAP (mmHg)</td>
</tr>
<tr>
<td>Diastolic dysfunction (%)</td>
</tr>
<tr>
<td>Left ventricle diastolic diameter (mm)</td>
</tr>
<tr>
<td>Left ventricle systolic diameter (mm)</td>
</tr>
<tr>
<td>Blood parameters:</td>
</tr>
<tr>
<td>Hemoglobin (mmol/L)</td>
</tr>
<tr>
<td>Fetal Hb (%)</td>
</tr>
<tr>
<td>Leucocytes</td>
</tr>
<tr>
<td>Glomerular filtration rate (ml/min)**</td>
</tr>
<tr>
<td>LDH (U/L 37°C)</td>
</tr>
<tr>
<td>Markers of cardiac stress:</td>
</tr>
<tr>
<td>BNP (pg/mL)</td>
</tr>
<tr>
<td>NT-Pro-BNP (pg/mL)</td>
</tr>
</tbody>
</table>

*P<0.05 PHT versus no PHT. **Cockcroft and Gault-formula (males: 1.23 x weight x (140-age) / serum creatinine, females: 1.03 x weight x (140-age) / serum creatinine).
Trans-thoracic echocardiography
Trans-thoracic echocardiography was performed in 78 of the 85 included patients as 7 patients failed to meet their appointment on several occasions. In total 25 (32%) patients had PHT (see Table 1). Only two patients had moderate-severe PHT in the HbSS/HbSβ⁰-thalassemia group. Diastolic dysfunction occurred more frequently in patients with PHT although this did not reach statistical significance (P=0.136). No intra-cardiac shunts or right ventricular outflow obstruction were detected. Fourteen patients in the HbSS/Hbβ⁰-thalassemia group and 7 in the HbSC/HbSβ⁺-thalassemia group had no detectable tricuspid regurgitation jet flow. These patients did not differ in sex ratio or age from the other patients. As the number of patients with PHT in the HbSC/HbSβ⁺-thalassemia group was very low statistical analysis of comparisons between patients with and without PHT was not performed within this group.

<table>
<thead>
<tr>
<th>Table 2. Multi-variate regression analysis of logNT-proBNP.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall model without TRV⁸</td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>Overall model including TRV⁸</td>
</tr>
<tr>
<td>Independent variable</td>
</tr>
<tr>
<td>Genotype‡</td>
</tr>
<tr>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>Hemoglobin level</td>
</tr>
<tr>
<td>TRV</td>
</tr>
</tbody>
</table>

⁸Independent variables are genotype, hemoglobin level and glomerular filtration rate. *Standardized coefficient Beta. ‡HbSC/HbSβ⁺-thal (coded as 1) compared to HbSS/HbSβ⁰-thal (coded as 0). Adding usage of hydroxyurea to the model did not change these results.
Laboratory testing

Results for standard laboratory testing are presented in table 1. With all patients analyzed together, a modest but statistically significant correlation between TRV and NTproBNP levels was found ($r_s=0.292$, $P=0.015$) (see figure). However, no correlation could be demonstrated between TRV and BNP or NT-proBNP levels when analyzing HbSS/HbSβ0-thalassemia and HbSC/HbSβ+-thalassemia patients separately. If a previously defined cut-off value of 160pg/mL was used, only 28% of patients with PHT had an elevated NT-proBNP level (sensitivity 28%), as opposed to 14% (specificity 86%) of non PHT patients ($P=0.159$). Corresponding negative and positive predicting values were 74% and 46%, respectively. Patients with trace or no tricuspid regurgitation flow had comparable NT-proBNP and BNP plasma levels as compared to patients with a detectable TRV < 2.5 m/s. NT-proBNP and BNP plasma levels correlated inversely with hemoglobin levels ($r_s= -0.435$; $P= 0.002$ in the HbSS/Sβ0-thalassemia group and $r_s= -0.449$; $P=0.032$ in the HbSC/HbSβ+-thalassemia group). BNP levels correlated significantly only with hemoglobin level in the HbSS/Sβ0-thalassemia group ($r_s= -0.624$; $P<0.001$). Both hemoglobin concentrations and the glomerular filtration rate correlated significantly with log transformed NT-proBNP in multi-variate regression analysis. Adding TRV to the model did not alter the model coefficients (see table 2). No significant relation between diastolic dysfunction and NT-proBNP or BNP levels could be demonstrated. Adding hydroxyurea therapy did not alter the results of the applied multi-variate model regarding (NT-pro)BNP levels.

Chest x-rays

Chest x-rays were performed in 63 patients with echocardiographic results (22 with and 41 without PHT). There was no significant difference in cardiothoracic ratio (CTR) between the patients with and without PHT, with CTR significantly related to hemoglobin ($r=-0.556$; $P<0.001$) and lactate dehydrogenase (LDH) ($r=0.494$; $P<0.001$) concentrations, as well as BNP ($r=0.418$; $P=0.001$) and NT-proBNP ($r=0.367$; $P=0.004$) plasma levels. Similar results were obtained when analyzing HbSS/HbSβ0-thalassemia patients alone, but not in HbSC/HbSβ+-thalassemia patients (data not shown).
High resolution computerized tomography

After performing 51 HRCTs we stopped performing these due to the very low prevalence of radiological abnormalities indicative of risk factors for PHT. There were no differences in the included genotypes, clinical history, PHT prevalence or age between the patients that underwent HRCT scanning as compared to those who did not. The prevalence of all pre-defined abnormalities on HRCT was low. None of the patients had signs of honeycombing, pleural effusion, acinar nodules, pericardial effusion, pericardial thickening or septum bulging on their HRCT scan. Since the extent of abnormalities on HRCT was low only the most affected lung quadrant was used for comparative analysis. In the HbSS/HbSβ⁰-thalassemia group no differences between patients with and without PHT were detected on HRCT (see table 3).

Table 3. Prevalence of any CT abnormalities.

<table>
<thead>
<tr>
<th></th>
<th>HbSS/Sβ⁰-thal</th>
<th>HbSC/Sβ²-thal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHT</td>
<td>No PHT</td>
</tr>
<tr>
<td>Number of patients</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>Bronchial thickening</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Bronchiolocasting</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Mosaic perfusion</td>
<td>1 (18%)</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Groundglass opacities</td>
<td>4 (16%)</td>
<td>9 (8%)</td>
</tr>
<tr>
<td>Consolidation</td>
<td>1 (1%)</td>
<td>5 (3%)</td>
</tr>
<tr>
<td>Reticular density</td>
<td>7 (10%)</td>
<td>14 (5%)</td>
</tr>
<tr>
<td>Subpleural lines†</td>
<td>3 (mild)</td>
<td>6 (mild)</td>
</tr>
<tr>
<td>Lobar volume loss†</td>
<td>2 (mild)</td>
<td>3 (mild)</td>
</tr>
<tr>
<td>Thickened interlobar</td>
<td>5 (mild)</td>
<td>14 (mild)</td>
</tr>
<tr>
<td>Parenchymal bands†</td>
<td>3 (mild)</td>
<td>6 (mild)</td>
</tr>
<tr>
<td>Nodules‡</td>
<td>1 (mild)</td>
<td>3 (mild)</td>
</tr>
<tr>
<td>Pleural thickening‡</td>
<td>8 (mild)</td>
<td>11 (mild)</td>
</tr>
</tbody>
</table>

The first number denotes the number of patients with the specified radiological abnormality on HRCT. The second number (between brackets) denotes the median percentage of affected lung area or the most prevalent category. This second number includes only the patients that did have signs of the specified radiological abnormality. †Categories were none, mild, medium or severe (see supplemental data). No significant differences were found between patients with or without PHT.
Pulmonary function tests
Pulmonary function tests were performed in 74 patients (of whom 70 patients underwent echocardiography). Forty-one patients (55.4%) appeared to have restrictive pulmonary function while 4 patients (5.4%) had evidence of obstructive lung disease. Twenty-nine patients (39.2%) had normal pulmonary function tests. No statistically significant differences in pulmonary function tests were detected patients with or without PHT in the HbSS/Sβ⁰-thal patients (see table 4).

Electrocardiography
In total 74 electrocardiograms were available for analysis (of whom 70 patients underwent echocardiography). In the HbSS/Sβ⁰-thal patients, the median R-axis (degrees) in those with PHT and without PHT was respectively 26 (12-49) and 43 (29-54) (P>0.05). In HbSC/HbSβ⁺-thal patients, the median R-axis (degrees) in those with PHT and without PHT was respectively 18 (-5-42) and 46 (12-52) (P>0.05). None of the patients had a right-axis deviation or a P-pulmonale. Two patients had a R-wave in V1 larger than 7 mm, one of which had PHT. Comparison of all other electrocardiographic parameters between patients with or without PHT did not show any significant difference.

<table>
<thead>
<tr>
<th>Table 4. Results spirometry and plethysmography.</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td><strong>HbSS/Sβ⁰-thal</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Number of patients</strong></td>
</tr>
<tr>
<td><strong>FVC</strong></td>
</tr>
<tr>
<td><strong>FEV₁</strong></td>
</tr>
<tr>
<td><strong>FEV₁/FVC</strong></td>
</tr>
<tr>
<td><strong>Tl,CO SB</strong></td>
</tr>
<tr>
<td><strong>Tl,CO SB VA</strong></td>
</tr>
<tr>
<td><strong>VA</strong></td>
</tr>
<tr>
<td><strong>TLC</strong></td>
</tr>
</tbody>
</table>

Numbers are percentage of expected following ATS/ERS standard and predicted values.³ VFC= forced vital capacity; FEV₁= forced expiratory volume in one second; TLC= total lung capacity; Tl,CO SB=Transfer factor for carbon monoxide using the single-breath gas transfer technique (corrected for Hemoglobin); Tl,CO SB VA= Tl,CO corrected for alveolar volume; VA= Alveolar volume.
Discussion

The objective of our study was to assess the value of an array of cardiopulmonary imaging and functional studies in relation to PHT in SCD. The studied cohort was prospectively screened for PHT employing echocardiography. The prevalence of PHT was comparable to that reported by others.\textsuperscript{1-2,13} As was recently reported\textsuperscript{14}, diastolic dysfunction occurred more frequently in patients with PHT, although the difference did not reach statistically significance in our group.

Several forms of interstitial lung disease are associated with PHT. In SCD scarring and fibrosis occurs secondary to lung infarction. In our cohort, 51 HRCTs were systematically evaluated and demonstrated only minor parenchymal abnormalities irrespective of ACS history which is in line with a previous HRCT study in SCD.\textsuperscript{3} The prevalence and the extent of mosaic patterns, ground glass opacities or other abnormalities did not differ between patients with and without PHT. Of interest, the artery to bronchus ratio was not associated with the presence of PHT in our patients. HRCT abnormalities were largely undetectable with standard chest x-rays. Parenchymal lung disease does therefore not seem to be causally related to PHT in SCD.

Both restrictive and obstructive pulmonary disease occurs in a significant number of sickle cell patients and are recognized causes of PHT in the non SCD population.\textsuperscript{15-16} Pulmonary function did not differ between patients with and without SCD-associated PHT in our study confirming previous work\textsuperscript{2d}. However, a recent case control study demonstrated lower lung volumes in 26 HbSS patients with PHT as compared to 17 HbSS patients without PHT with a comparable transfer factor for carbon monoxide.\textsuperscript{9} Contrary to our cohort these patients were characterized by moderate-severe PHT. Taken together, these data argue against a role of primary importance of either restrictive or obstructive pulmonary disease in the etiology of SCD-related PHT (even though no broncho-provocation testing was performed).

Systemic evaluation of ECGs in all patients showed no signs of right ventricular dilatation or hypertrophy or P-pulmonale in any of the patients with PHT and ECG findings did not differ between patients with and without PHT. Although such ECG changes have been described in patients with other forms of PHT, the lack of these specific ECG findings in our patients may be explained by the relatively low systolic sPAP in our cohort and by the fact that more subtle changes may have been masked by ECG changes due to left ventricle enlargement.\textsuperscript{10} Therefore, ECGs are not suitable as a screening tool for mild SCD related PHT.

Plasma NT-proBNP and BNP levels correlate significantly to TRV in patients with idiopathic PHT and two recent studies have shown plasma NT-proBNP and BNP levels to be related to PHT in SCD,\textsuperscript{11,12} indicating that plasma (NT-pro)BNP or BNP levels may be valuable tools for screening for (the development of) PHT in SCD. However, in our cohort consisting almost exclusively of patients with mild PHT, no statistically significant correlation could be detected between (NT-
(NT-pro)BNP and TRV after correcting for genotype. Furthermore, in a separate multivariate regression analysis NT-proBNP levels appeared to depend solely on hemoglobin concentration and the GFR. Previous studies of (NT-pro)BNP in SCD analyzed all SCD genotypes together.\textsuperscript{11,12} As HbSC and HbS\textsuperscript{β}-thalassemia patients have a significantly lower rate of hemolysis, less impaired renal function and lower TRVs with a lower incidence of PHT, as compared to HbSS and HbS\textsuperscript{β}-thalassemia patients, this could in part explain the differences between these studies and our data. We cannot exclude, however, that the relatively lower number of patients in our study could also contribute. Nonetheless, based upon our data the most important determinant of plasma (NT-pro)BNP levels in SCD seems to be the degree of anemia and not the presence of mild PHT. Heart size, as estimated with the CTR, was indeed significantly related hemolysis rate but not to the presence of PHT or tricuspid regurgitation flow velocity. In addition, renal dysfunction leads to higher plasma (NT-pro) BNP levels and the degree of anemia has also shown to be related to the presence of renal dysfunction in SCD.\textsuperscript{13} In this light, two recent studies demonstrated that the prognostic significance of NT-proBNP levels correlated with renal function in patients with heart failure and appeared to have no prognostic value after correction for blood urea nitrogen levels.\textsuperscript{14,15} At this time, plasma (NT-pro) BNP levels cannot be recommended tools for cross sectional PHT screening.

**Figure.** Scatterplot showing the relation between NT-proBNP and tricuspid regurgitation jet flow velocity.
In interpreting these data a few aspects need to be considered. Firstly, since our study was performed in three sickle cell centers, referral bias may be present. However, most sickle cell patients in the Netherlands are either cared for at referral centers or visit a referral center at regular intervals. Therefore, referral bias is likely limited. Secondly, 27% of patients had only a trace or no detectable TRV and PHT might have been missed. However, previous studies found a very low mortality in these patients, arguing against a high prevalence of PHT in this group.\textsuperscript{1} Thirdly, this cohort included relatively few patients with moderate-severe PHT, limiting the applicability of our findings to sickle cell patients with mild PHT. However, as in previous studies, this is the largest group detected with echocardiographic screening and, importantly, mortality is high in these patients as well.\textsuperscript{1,3}

In conclusion, once a sickle cell patient is diagnosed with mild PHT (e.g. a TRV 2.5-2.9m/s), additional diagnostic testing with chest X-ray, HRCT of the thorax, pulmonary function testing and ECG do not seem to offer clinically relevant information with regard to the etiology of SCD related PHT, nor do they offer clues to the presence of mild PHT in SCD. Also, based upon our findings, plasma (NT-pro)BNP levels cannot be recommended as a sole screening tool for detecting mild PHT in SCD.
Supplement

High Resolution Computerized tomography

High Resolution Computerized Tomography (HRCT) scans were obtained in the supine position, at full inspiration, with 1.5 mm collimation at 10 mm intervals. Images were transferred in a central picture archiving and communication system (IMPAX) for clinical interpretation. All HRCT images were centrally reviewed by an experienced observer blinded to clinical data. HRCT scores were determined using a modification of a previously described technique.²² Before analysis the lungs were divided in an upper and a lower part (respectively above and below the bifurcation of the trachea) creating four lung quadrants. Since the extent of HRCT abnormalities was generally low, only the most affected lung quadrant was used for further data analysis.

Separately in each quadrant, HRCT abnormalities were quantified as follows: A) volume loss (0=none; 1=mild; 2=intermediate; 3=severe); B) prominence of (centrilobular) nodules (0=none; 1=mild; 2=intermediate; 3=severe); C) the prominence of pleural thickening (0=none; 1=mild; 2=intermediate; 3=severe); D) the extent of thickened interlobular septa (0=none; 1=less than five interlobular septa; 2=more than five interlobular septa but less than 50% involvement of the pleural surface; 3= more than 50% involvement of the pleural surface) and E) the presence of parenchymal bands (0=none; 1=less than five parenchymal bands; 2=more than five parenchymal bands); F) the presence of subpleural lines (0=none; 1=mild; 2=intermediate; 3=severe). In the summary of the HRCT data in table 2 of the main article all categories are simplified in 0=none; 1=mild; 2=medium; 3=severe.

In addition, specific pulmonary patterns were scored on a continuous scale to the nearest 5%: A) a reticular pattern (defined as innumerable interlacing line shadows suggesting a mesh); B) groundglass opacification (defined as a hazy increased attenuation of lung with preservation of vascular and bronchovascular markings); C) mosaic pattern (defined as areas of hazy decreased attenuation versus areas of relatively increased attenuation, sharply demarcated against each other by interlobar septa); D) consolidation; E) honey combing; and F) ill defined centrolobular acinar nodules. Furthermore, the presence of tractional dilatation of segmental and subsegmental airways (respectively traction bronchiectasis and bronchioliecctasis), pleural effusion and bronchial wall thickening was recorded.

Next, in each lung quadrant, the ratio of the artery to bronchus diameter was visually estimated; this was done separately in the central perihilar area, in the midportion of the lung and in the peripheral subpleural region. These locations were aimed to correspond to the lobar, segmental and subsegmental level of the arteries and bronchi, respectively. An increase of this ratio has previously been reported as an indicator of pulmonary hypertension.¹⁷ Care was taken that the AB-ratio was
not affected by branching of the artery or bronchus, respectively, at different locations. To take into account a rather broad anatomic variation of the AB ratio we coded a ratio < 1 as 0, a ratio of 1-1.5 as 1, a ratio of 1.5-2 as 2 and a ratio exceeding 2 as 3.

The heart was examined for signs of pericardial effusion, pericardial thickening, septum bulging and right ventricle enlargement. Finally, the vertebrae, ribs and scapulas were evaluated for signs of osteonecrosis by another radiologist specialized in imaging of the musculoskeletal system (more than 15 years of experience).
Reference List


Dissociation of exercise tolerance and pulmonary hypertension in patients with sickle cell disease

(Submitted)

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⁴Department of Pulmonology, VU Medical Center, Amsterdam.
Abstract

Pulmonary hypertension (PH) is observed in ±30% of adult sickle cell disease (SCD) patients and is associated with poor prognosis. The aim of this study was to assess whether PH is implicated in exercise intolerance in SCD patients.

We performed pulmonary function tests and symptom limited cardiopulmonary exercise testing in consecutive adult SCD patients screened for pulmonary hypertension by echocardiography. Forty-one patients with SCD were studied. Ten patients had evidence for PH at rest. Exercise capacity was decreased in 34(83%) of the studied patients. After correction for haemoglobin concentration, peak oxygen uptake (VO₂-peak) did not differ between patients with and without PH. Multivariate linear regression analysis showed haemoglobin concentration and vital capacity to be independently associated with VO₂-peak. No relation was found between PH and exercise performance. According to criteria for exercise limitation, only one patient had pulmonary vascular exercise limitation.

In unselected adult SCD patients reduced exercise capacity was found to be determined by anaemia and pulmonary function impairment, rather than the presence of pulmonary hypertension. This suggests that rather than being an autonomous complication of SCD, the presence of mild PH should be considered a marker of disease severity in these patients.
Introduction

Sickle cell disease (SCD) is characterized by chronic haemolytic anaemia and (micro)vascular occlusion, resulting in accumulating organ damage, a decreased quality of life and a reduced life expectancy. Pulmonary hypertension (PH) is a recently recognized SCD-associated complication. PH is reported to be present in approximately 30% of adult sickle cell anaemia patients and has been identified as an independent risk factor for early death.\textsuperscript{1,2} Importantly, even mild PH appears associated with a short-term increased risk of death. Since PH-related clinical signs and symptoms in these patients can be absent or obscured by other disease manifestations, it is generally recommended to screen all adult SCD patients for the presence of PH by use of transthoracic echocardiography.\textsuperscript{2} Moreover, management guidelines for treatment of PH in SCD patients have been published.\textsuperscript{3} A reduced exercise capacity is one of many manifestations of PH; and exercise capacity, reflected by peak oxygen consumption (V'O\textsubscript{2-peak}) in a symptom limited cardiopulmonary exercise test (CPET), is an important predictor of survival in various forms of PH.\textsuperscript{4} In SCD, however, anaemia, restrictive and obstructive pulmonary disease,\textsuperscript{5,6} cardiomyopathy and skeletal damage may all contribute to a reduced exercise capacity. The contribution of mild PH to the reduced exercise capacity in SCD has not been extensively studied. As PH results in a typical pattern of physiological changes during CPET, we set out to investigate to which extend PH contributes to the exercise intolerance in SCD patients. Therefore, we performed pulmonary function tests and CPET in consecutive adult SCD patients screened for pulmonary hypertension. The aim of this study was to assess whether PH is implicated in exercise intolerance in these patients.
Methods

Subjects
Adult sickle cell patients (HbSS, HbSC, HbSβ+ -thalassemia or HbSβ₀-thalassemia, confirmed by high performance liquid chromatography), who visited the outpatient haematology clinic of the Academic Medical Centre (AMC) for routine scheduled evaluation were considered eligible for this study. Inclusion criteria were: age 18 years or older and the physical capacity for CPET. Exclusion criteria were: pregnancy, a recent vaso-occlusive crisis (< 2 weeks before) or acute chest syndrome (< 4 weeks before). The genotypes associated with more severe disease (HbSS and HbSβ₀-thalassemia) were grouped together for analysis, as were the relatively milder disease genotypes (HbSC and HbSβ+ -thalassemia). All patients gave written informed consent. The research protocol was approved by the local institutional review board and the study was carried out in accordance with the principles of the declaration of Helsinki.

Study design
This was a controlled, cross-sectional study. Patients with PH, defined according to generally used criteria in SCD (see below), were compared with SCD patients without evidence for PH. The study consisted of two visits. During the first visit, after giving informed consent, patients underwent transthoracic echocardiography, laboratory testing and pulmonary function testing. A symptom limited CPET was performed during a second visit, within 2 weeks after the first visit.

Trans-thoracic echocardiography
Trans-thoracic echocardiography was performed as described before.⁷ According to the generally used SCD-specific criteria, the presence of PH was defined as a peak tricuspid regurgitation jet flow velocity (TRV) of at least 2.5 m·sec⁻¹ (corresponding to an estimated systolic pulmonary artery pressure of greater than or equal to 30 mmHg). This measurement has been reported to correlate with sPAP in the absence of right ventricular outflow obstruction and pulmonary stenosis and has been validated in patients with SCD.² Patients with a TRV 2.5-2.9 m·sec⁻¹ are considered to have mild PH, whereas patients with a TRV ≥3.0 m/s in general suffer from moderate-to-severe PH.² Patients with trace or no tricuspid regurgitant were considered to have normal sPAP with the TRV assigned a value of 1.3 m·sec⁻¹.⁸
Laboratory testing
Venous blood samples for baseline laboratory testing (complete blood count, Hb-electrophoresis, creatinin, urea, LDH, bilirubin, NT-pro BNP) were drawn at rest and directly measured according to local protocols including HLPC of EDTA coagulated blood to determine the percentage of foetal haemoglobin (HbF). NT-proBNP was measured in EDTA-anticoagulated plasma with an electro-chemiluminescence immunoassay (Roche Diagnostics).

Pulmonary function testing
Spirometry was performed by pneumotachography (Jaeger MS diffusion, Wuerzburg, Germany) and included forced vital capacity (FVC), forced expiratory volume in one second (FEV1) and inspiratory slow vital capacity (IVC). Total lung capacity (TLC) was determined by plethysmography (Jaeger MS Body, Wuerzburg, Germany). Transfer factor for carbon monoxide (Tl,CO), pulmonary capillary blood volume (Qc) and pulmonary membrane diffusion capacity (Dm) were determined from measurements of Tl,CO at high (±92%) and low (±19%) oxygen concentration, using the single breath technique.9 Tl,CO was corrected for haemoglobin concentration according to ATS/ERS criteria.10 Spirometry, plethysmography and Tl,CO were measured according to the ATS/ERS guidelines.10-12

Cardiopulmonary exercise testing
Symptom limited CPET was performed according to the guidelines of the American Thoracic Society.13 Briefly, following placement of a radial artery catheter for arterial blood sampling, patients were placed on a cycle ergometer in the upright position and continuous measurements were made of minute ventilation (Ve), VO2, VCO2, heart rate, blood pressure and electrocardiography. Work load was increased by 5 to 15 Watt, depending on the predicted maximum exercise capacity and in such a way that maximal effort was attained within 10-15 minutes. Every two minutes arterial blood gas analysis was performed. Exercise limitation was assessed according to the guidelines of the American Thoracic Society.13 (Table 1) The PH specific pattern of physiological changes during CPET is characterized by reduced peak VO2 (< 84% predicted), low VO2 at the anaerobic threshold (VO2@AT; < 40% predicted), normal to high ventilatory reserve (pre-assessed maximal ventilation minus actual peak-ventilation; > 11 L or > 15%) and a high ventilatory equivalent for CO2 (Ve/V'CO2) at the anaerobic threshold (< 34).14-17
Statistical analysis

Data are expressed as mean ± standard deviations (SD). All analyses were performed with the SPSS statistical package (SPSS 13.0; Chicago, IL). Pearson’s correlation coefficient was calculated for correlation studies, and was tested for two-sided significance. Multivariate linear regression analysis of all individual parameters that correlated significantly with either TRV or exercise capacity was performed to calculate their predictive value in relation to TRV or exercise capacity. A paired t-test was used to analyze between group differences, except for laboratory testing, for which the Mann-Whitney test was employed. In case of differences in baseline characteristics, a subgroup analysis was planned between patients with increased TRV and matched controls. A Chi-squared test was used to compare PH prevalence between the mild and severe genotype group. A p-value <0.05 was considered statistically significant

<table>
<thead>
<tr>
<th>Variables</th>
<th>Criteria of normality</th>
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</thead>
<tbody>
<tr>
<td>VO₂ max or VO₂ peak</td>
<td>&gt; 84% predicted</td>
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<tr>
<td>Anaerobic threshold</td>
<td>&gt; 40% VO₂ max predicted</td>
</tr>
<tr>
<td>Heart rate max</td>
<td>&gt; 90% predicted</td>
</tr>
<tr>
<td>Heart rate reserve</td>
<td>&lt; 15 beats/min</td>
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<tr>
<td>Ventilatory reserve</td>
<td>&gt; 11 L or &gt; 15%</td>
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<tr>
<td>O₂-pulse (VO₂/HR)</td>
<td>&gt; 80%</td>
</tr>
<tr>
<td>V'/VCO₂ @ AT</td>
<td>&lt; 34</td>
</tr>
<tr>
<td>VD/VT</td>
<td>&lt; 0.28 or &lt;0.30 for age &gt;40 years</td>
</tr>
<tr>
<td>PaO₂</td>
<td>&gt; 10.7 kPa</td>
</tr>
<tr>
<td>P(A-a)O₂</td>
<td>&gt; 4.7 kPa</td>
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</tbody>
</table>

Table 1. ATS suggested normal guidelines for interpretation of cardiopulmonary exercise testing results.¹³
Results

Patients
Fifty-one SCD patients were eligible for this study. Ten patients were excluded: two because of a sickle cell crisis shortly before testing, 3 patients were not able to perform CPET because of knee joint problems and 5 patients refused to participate. Except for age (28.7 ± 10.7 years vs. 42.1 ± 13.7 years p<0.001), baseline characteristics did not differ between the included and excluded patients. During routinely screening, two of the excluded patients were found to have PH, with a TRV of 2.5 and 2.8 m·sec⁻¹, respectively. Baseline characteristics and laboratory data are summarized in Table 2.

Trans-thoracic echocardiography
Ten patients (24%; 5 male, 5 female, mean age 31 ± 12.1 y) had evidence for PH at rest, i.e. a TRV≥2.5 m·sec⁻¹. Of the 31 patients without PH, 8 had an undetectable TRV. Except for haemoglobin concentration, there was no difference in baseline characteristics or laboratory parameters between patients with or without PH (Table 3). The prevalence of PH did not differ significantly between the relatively milder and more severe genotype group (3 PH out of 15 HbSC/HbSβ⁺ patients versus 7 PH out of 26 HbSS/HbSβ⁰ patients; p=0.62).

Pulmonary function
Results of pulmonary function testing are shown in Table 3. On average, FVC, FEV₁ and TLC were relatively low. A restrictive ventilatory defect, with a TLC below the 5th percentile of the predicted value, was present in 22 patients, while 3 patients had a mild obstructive ventilatory defect. Average TLCO was decreased, but normal if TLCO was corrected for the level of restriction. PH patients had significantly lower VC, FEV₁ and TLC as compared to patients without PH. Moreover, VC and FEV₁ were significantly correlated to the haemoglobin concentration (VC r=0.41; FEV₁ r=0.48; both p<0.05). After correction for the haemoglobin level no significant difference in pulmonary function parameters was found between the patients with and without PH.

### Table 2. Baseline characteristics.

<table>
<thead>
<tr>
<th>Patient characteristics:</th>
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<tbody>
<tr>
<td>Age (yrs)</td>
<td>29 (17-54)*</td>
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<tr>
<td>Gender (M/F)</td>
<td>13/28</td>
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<tr>
<td>HbSS</td>
<td>22</td>
</tr>
<tr>
<td>HbSC</td>
<td>12</td>
</tr>
<tr>
<td>HbSβ⁺</td>
<td>3</td>
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<tr>
<td>HbSβ⁰</td>
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</table>

<table>
<thead>
<tr>
<th>Laboratory parameters:</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Hemoglobin (mmol/l)</td>
<td>6.1 ± 1.1</td>
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<tr>
<td>NT-Pro BNP (ng/l)</td>
<td>94 ± 116</td>
</tr>
</tbody>
</table>

Pulmonary function parameters (% predicted):

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVC</td>
<td>83.3 ± 14.7</td>
</tr>
<tr>
<td>FEV₁</td>
<td>81.8 ± 15.6</td>
</tr>
<tr>
<td>TLC</td>
<td>80.2 ± 12.1</td>
</tr>
<tr>
<td>TLCO</td>
<td>74.5 ± 13.1</td>
</tr>
<tr>
<td>TLCO/Va</td>
<td>100.8 ± 20.2</td>
</tr>
</tbody>
</table>

*median (range)
Cardiopulmonary exercise testing

Thirty-four of the 41 studied patients (83%) had a diminished exercise capacity with a decreased peak oxygen uptake (V\text{O}_2\text{-peak}), i.e. below 84% of the predicted value. In PH patients as compared to patients without PH, mean V\text{O}_2\text{-peak} and mean oxygen uptake at the anaerobic threshold (V\text{O}_2@AT) were lower, however, these differences did not reach statistical significance (63.5 ± 12.6% versus 73.4 ± 16.4%, p= 0.097, and 48.7± 10.2% versus 57.0± 17.6%, p=0.17, respectively;).

| Table 3. Patient characteristics, laboratory parameters, pulmonary function parameters. |
|-------------------------------------------------|-----------------|-----------------|
| Patient characteristics:                        | TRV < 2.5 m/s   | TRV ≥2.5 m/s    |
| N                                               | 31              | 10              |
| Age, yrs                                        | 29 ± 11         | 32 ± 12         |
| Gender, M/F                                     | 8/23            | 5/5             |
| HbSS                                            | 15              | 7               |
| HbSC                                            | 10              | 2               |
| HbS§²                                           | 2               | 1               |
| HbS§³                                           | 4               | 0               |
| Laboratory parameters:                          |                 |                 |
| Hemoglobin (mmol/l)                             | 6.2 ± 1.0       | 5.3 ± 1.1*      |
| Fetal Hb (%)                                    | 8.3 ± 8.6       | 8.7 ± 6.6       |
| Kreatinin (umol/l)                              | 54.1 ± 14.3     | 130.1 ± 220.7   |
| Direct Bilirubin (umol/l)                       | 11.8 ± 26.7     | 9.8 ± 11.0      |
| Total Bilirubin (umol/l)                        | 55.9 ± 53.5     | 56.8 ± 44.3     |
| Urea (mmol/l)                                   | 2.88 ± 0.79     | 4.81 ± 4.88     |
| LDH (u/l)                                       | 346.8 ± 147.2   | 355.0 ± 204.0   |
| NT-Pro BNP (ng/l)                               | 82.3 ± 111.3    | 135.8 ± 131.8   |
| Pulmonary function parameters (% predicted):    |                 |                 |
| FVC                                             | 86.1 ± 14.5     | 74.8 ± 12.3*    |
| FEV\textsubscript{1}                            | 84.7 ± 15.8     | 72.8 ± 11.3*    |
| TLC                                             | 82.7 ± 11.2     | 72.3 ± 12.0*    |
| T\textsubscript{L,CO}                           | 75.8 ± 11.8     | 70.7 ± 16.5     |
| T\textsubscript{L,CO}/V\textsubscript{A}       | 98.9 ± 19.0     | 106.8 ± 23.7    |
| V\text{O}_2\text{-peak}                        | 73.4 ± 16.9     | 63.5 ± 12.6     |
| V\textsubscript{E}/V\text{CO}_2@AT **           | 28.5 ± 4.0      | 27.6 ± 3.4      |

*significantly different from TRV< 2.5 m/s (p-value <0.05) **ratio.
However, as there was a significant correlation between V'O₂_peak and haemoglobin concentration (r=0.46, p<0.005; Figure 2), correction for haemoglobin concentration fully levelled out these small differences. Moreover, V'E/V'CO₂ at anaerobic threshold, reported to be increased in various forms of PH,¹⁴-¹⁶,¹⁸ was not elevated in the PH patients as compared to patients without PH (Figure 1). Multivariate linear regression analysis demonstrated haemoglobin concentration and VC to be independently associated with V'O₂_peak. (Model: r²=0.45, p<0.001; standardized β=0.48, p<0.001 and 0.31, p<0.05 respectively). When included in the model, genotype did not predict V'O₂_peak. Moreover, no significant correlation was found between TRV and exercise performance. Reduced exercise capacity met the employed criteria for exercise limitation by pulmonary vascular limitation in one patient only (HbSS with PH). Based on CPET criteria,¹⁷ the other patients were limited in exercise capacity due to anaemia (n=17), cardiovascular dysfunction (n=2), musculoskeletal function (n=10), pulmonary function abnormalities (n=1), and poor effort (n=3).
Discussion

In this study, we assessed by use of cardiopulmonary exercise testing to what extent pulmonary hypertension contributes to the decreased exercise capacity that can be observed in adult sickle cell patients. Thirty-four out of 41 evaluated patients had a decreased exercise capacity, 24% of whom had evidence for mild PH at rest. Exercise limitation observed in the studied patients could not be attributed to a pulmonary vascular limitation due to pulmonary hypertension. Multivariate linear regression analysis showed anaemia and pulmonary function impairment to be the most predominant factors contributing to the reduction in exercise capacity. This suggests that rather than being an autonomous complication of SCD, the presence of mild PH should be considered a marker of disease severity in these patients.

According to the generally accepted criteria for interpretation of clinical exercise testing, pulmonary vascular limitation is likely when \( V'\text{O}_2\text{-peak} \) and \( V'\text{O}_2 \) at the anaerobic threshold are reduced, breathing reserve is normal or high, and \( V'\text{E}/V'\text{CO}_2 \) at anaerobic threshold is increased.\(^{14-17}\) Individual interpretation of all exercise tests in our study showed a pulmonary vascular limitation in only one sickle cell patient. As anaemia was the most common cause of exercise limitation in our patients, it is not surprising that \( V'\text{O}_2\text{-peak} \) was significantly related to haemoglobin concentration.

A previous study using CPET in 19 selected female SCD patients demonstrated pulmonary vascular limitation in 11 patients whereas anaemia was the sole cause of exercise limitation in only 3 patients.\(^{19}\) In this study, however, neither the prevalence nor the severity of PH was reported. Therefore, it remains unclear whether the reported pulmonary vascular limitations were related to this complication.

Furthermore, Anthi and co-workers recently reported a significantly lower exercise capacity in SCD patients with PH as opposed to patients without PH matched for haemoglobin concentration.\(^{20}\) In our view, this apparent discrepancy can be explained at least in part by the fact that a highly selected and older patient population was studied, characterized by moderate-to-severe PH, whereas our consecutively included patients were almost exclusively characterized by evidence for mild PH at the most. Interestingly, Anthi et al. also reported that 50% of their PH patients appeared to have an elevated TRV secondary to pulmonary venous hypertension.\(^{20}\) In SCD, left ventricular hypertrophy is inversely related to the degree of anaemia, and an increased left ventricular mass was demonstrated to be positively correlated with right ventricular pressures.\(^{21}\) Taken together, it may be suggested that, at least in part of the SCD patients, PH is secondary to anaemia-induced left ventricular hypertrophy. As both heart and lung are trapped in the narrow space of the thorax, an increase in heart size may lead to a decrease in lung volume. This mechanism might explain the relation between the pulmonary function parameters and exercise capacity, as well as the decreased lung volumes in PH patients in the present study. As such, a restrictive pulmonary function defect, a reduced exercise capacity, evidence for PH and anaemia may all represent causally related markers.
of severity of disease in SCD patients. At the same time, it might also be hypothesized that the degree of pulmonary hypertension in our patients was too mild to cause a significant reduction in exercise tolerance. This is supported by the findings of Machado et al. on the effect of open-label use of sildenafil on exercise tolerance in SCD patients with PH, measured by the 6-minute walk test. Although sildenafil improved exercise tolerance in all studied patients, no decrease in estimated systolic PAP could be demonstrated in patients with mild PH (TRV < 3.0 m·sec⁻¹), suggesting that sildenafil may have improved exercise tolerance in this subset of patients by another mechanism, that is an increased ventilatory efficiency or an improved cardiac function.

A few methodological aspects of our study need comment. Firstly, the relatively small number of patients needs to be taken into account and these findings need to be confirmed in a larger cohort. Secondly, right heart catheterization remains the gold standard diagnostic test for PH and is indeed recommended in SCD patients with moderate-to-severe PH. The criteria used in this study to define the presence of PH are generally used and validated in SCD patients. However, in view of the relatively low specificity of the used cut-off level, by doing so we may have included patients without pulmonary hypertension. Since we wished to study whether exercise limitation in SCD patients could be attributed to a pulmonary vascular limitation due to the presence of pulmonary hypertension, we do not feel that the lack of right heart catheterization would significantly affect our present results.

In conclusion, in the present study we demonstrate that anemia and pulmonary function impairment, rather than pulmonary hypertension is responsible for the reduced exercise tolerance in unselected sickle cell patients. These findings do not support a direct causal relationship between the pulmonary hypertension and the increased short-term mortality observed in SCD patients with mild pulmonary hypertension only. Based upon our findings it can be argued that the presence of pulmonary hypertension in these SCD patients reflects the severity of the disease. As such, however, our findings underline the necessity to screen all patients with SCD for the presence of PH, irrespective of the clinical presentation.
Acknowledgements

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Reference List


Chapter 11


Summary

Samenvatting

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Summary

Only fifty years ago sickle cell disease (SCD) was regarded mainly as a disease of childhood. By introducing new therapies and improving standard of care for children with SCD most patients will nowadays reach adulthood in countries with appropriate medical resources. However, with the increased life expectancy accumulating organ damage throughout life is becoming a major concern. Next to suffering from acute complications such as vaso-occlusive sickle cell crises (considered the hallmark of SCD), acute chest syndromes and strokes, chronic complications such as renal failure, pulmonary hypertension, sickle retinopathy and iron overload are major determinants of SCD related morbidity and mortality in the ‘older’ patient.

In the first part of this thesis we studied clinical aspects of sickle cell disease. In the first chapter, a general overview of SCD is given, followed by a review of the current understanding of its pathophysiology. In chapter two, we describe the prevalence of various forms disease related complications in a representative patient cohort with SCD in the Netherlands. We conclude that several major forms of organ damage occur irrespective of the frequency of vaso-occlusive crises in adults with SCD, underlining the concept that vaso-occlusive crisis frequency, even though historically regarded as a major marker of disease severity, can not be used as a marker of disease severity in adult sickle cell patients in daily clinical practice. Since some forms of organ damage develop without clear symptoms, systematic screening for and evaluation of organ damage in all sickle cell patients seems indicated in order to initiate potential therapeutic measures as early as possible. In the third chapter, we compared continuous infusion of morphine to patient controlled analgesia (PCA) for the treatment of pain during vaso-occlusive crisis in a randomized controlled trial. We demonstrate that the use of PCA in patients with SCD during vaso-occlusive crisis results in a significant reduction in morphine consumption with equivalent response on measurements of pain and quality of life and a slight reduction in morphine-induced side-effects. For managing vaso-occlusive sickle cell crisis in the clinical setting, PCA with morphine should be favored over continuous morphine administration. However, future studies should address whether PCA is also superior to intermittent bolus opiate administration. The findings described above have already been incorporated in recently published guidelines for managing SCD in the Netherlands and Netherlands Antilles. Objective tools to diagnose a vaso-occlusive crisis or to quantify the extent of the microcirculatory obstruction during a vaso-occlusive crisis are currently not available but would be of value in the management of SCD for both diagnostic purposes and for monitoring effects of therapeutic interventions. In the study described in chapter four, we visualized the sublingual microcirculation and measured sublingual microvascular blood flow velocity in patients with SCD during vaso-occlusive crisis and during steady state with Side-stream Dark Field (SDF) imaging. In addition, healthy controls were analyzed. No differences in sublingual microvascular
blood flow velocity between sickle cell patients during vaso-occlusive crisis and steady state or between sickle cell patients and healthy volunteers could be detected. We conclude that sublingual microcirculatory blood flow velocity is not altered in sickle cell patients and is therefore not useful to monitor the systemic vaso-occlusive process in SCD.

In the second part of this thesis we explore pathophysiological aspects of SCD. Sickle cell disease (SCD) is characterized by a hypercoagulable state resulting from multiple factors, including chronic hemolysis and circulating cell-derived microparticles (MPs). There is no consensus on the cellular origin of such MPs and the exact mechanism by which they may contribute to coagulation activation in SCD is yet to be elucidated. In chapter five we determined the cellular origin of circulating MPs in patients with SCD during vaso-occlusive crises and in the clinically asymptomatic state, and explored their relation with coagulation, fibrinolysis and endothelial activation. Most circulating MPs were derived from erythrocytes and platelets, and the total number of MPs did not differ significantly between baseline conditions and crisis. Also, erythrocyte-derived MPs and in vivo coagulation, fibrinolysis and endothelial activation were significantly associated to each other. Furthermore, with thrombin generation experiments an almost 50% reduction in thrombin generation by anti-human factor XI was observed. These experiments imply an important role of factor XI-dependent thrombin generation by erythrocyte-derived MPs in SCD. The value of MP monitoring as surrogate parameter for assessing therapeutic interventions targeting especially the sickling process requires further investigation.

Another factor that may be involved in the pathophysiology of coagulation activation and the vascular microcirculation in SCD is disruption of the endothelial glycocalyx. New insights into endothelial biology have demonstrated that, in the quiescent state, the endothelium is shielded from circulating blood cells and proteins by the glycocalyx, a highly hydrated cell free mesh of membrane-associated proteoglycans, glycosaminoglycans, glycoproteins and glycolipids located at the endothelial surface. Over the last decades, it has become clear that endothelial activation and dysfunction play a central role in sickle cell vaso-occlusion. In chapter six, we demonstrate that sickle cell patients are characterized by a reduced glycocalyx volume. We hypothesize that glycocalyx perturbation could be a new factor of bi-directional importance in the complex pathophysiology of SCD, as glycocalyx volume reductions can both result from and contribute to vaso-occlusion. Although most studies regarding therapeutic interventions in SCD have measured therapy efficacy by the effect thereof upon vaso-occlusive crisis frequency, this is a poor marker of disease severity, as stated above. However, performing clinical trials with end points regarding developing organ damage will mostly not be feasible and therefore alternative objective tools of disease activity are direly needed. In this light the glycocalyx volume may proof to be a valuable tool for assessing the effects of therapeutic interventions in SCD.
The third part of the thesis specifically focuses on sickle cell related pulmonary hypertension (PHT), which is a recently recognized severe complication in SCD associated with a high risk of early death. The pathophysiology of SCD related PHT is largely unknown. However, an important role has been ascribed to a hemolysis induced reduction in nitric oxide (NO) bioavailability. It is of paramount importance to identify treatable causes and/or aggravating factors of PHT in SCD. Examples of other known causes and/or aggravating factors of PHT in general are: (in situ) pulmonary artery thrombosis, chronic obstructive lung disease, interstitial lung disease, and hypoxia induced vasoconstriction. All these factors occur in SCD but associations with PHT are scarcely reported. Also, elevated levels of asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase inhibitor, have recently been associated to PHT severity and outcome in both idiopathic and thrombo-embolic PHT, and ADMA levels are elevated in SCD. From a prospectively included patient cohort with mostly mild SCD-related PHT the following observations were made:

SCD related PHT is:

- common in patients with SCD in the Netherlands (chapter 2)
- not associated with vaso-occlusive crisis frequency (chapter 2)
- not caused by large to medium-sized pulmonary artery obstruction (chapter 7)
- not associated to the characteristic hypercoagulable state (chapter 8)
- not associated to parenchymal lung damage or abnormal pulmonary function testing (chapter 9)
- associated to plasma levels of asymmetric dimethylarginine (ADMA).

Even though several reports have shown PHT to be a risk factor for early death, it is unclear whether PHT itself is the direct cause of death or whether the presence of PHT should be regarded as a marker of general disease severity. As in general PHT is associated with a reduced exercise capacity, we compared symptom limited cardiopulmonary exercise testing (CPET) in patients with and without PHT. In only one patient the reduced exercise capacity could be explained by pulmonary vascular limitation while the majority of the studied patients was limited in exercise capacity because of anaemia, cardiovascular function, peripheral musculoskeletal function, pulmonary function abnormalities, or poor effort. We conclude that most unselected sickle cell patients with mild PHT have a reduced exercise capacity that appears to be determined by the severity of the anaemia and pulmonary function impairment, rather than the presence of PHT itself. These findings may cast at least some doubt as to whether is the PHT itself that translates to the high risk of early death. However, the facts that pulmonary artery pressures increase steeply during acute sickle cell related complications (such as acute chest syndrome or vaso-occlusive crises), that sudden death has been reported to occur in PHT patients during vaso-occlusive events and that
mild SCD-related PHT is also strongly associated with early death, do argue for a direct role of mild PHT in the increased mortality SCD.\textsuperscript{15}

The relation of ADMA to PHT in SCD further underlines the importance of a reduced NO bioavailability in SCD related PHT and future studies aimed at increasing NO bioavailability with respect to PHT development seem relevant. Also, the potential of plasma ADMA concentrations as a risk marker for the development of PHT (and other SCD related complications) is now subject of further study. From a practical viewpoint, screening sickle cell patients for PHT should solely be carried out by performing trans-thoracic echocardiography and additional work up, as recommended for PHT in general should be limited in sickle cell patients with mild PHT as the diagnostic yield will prove to be very low.
Samenvatting

Tot vijftig jaar geleden werd Sikkelenziekte (SCZ) voornamelijk beschouwd als een ziekte die vooral bij kinderen voorkomt. Door de introductie van nieuwe therapiën en met de verbeterde zorg voor kinderen met SCZ halen tegenwoordig de meeste patiënten in landen met een goede gezondheidsvoorziening wel de volwassen leeftijd. De verlengde levensverwachting zorgt echter wel voor een toename van morbiditeit in de vorm van chronische orgaanschade. Naast de acute complicaties, zoals de pijnlijke vaso-occlusieve crisis (waar SCZ zich door kenmerkt), het acuut chest syndroom (acute chest syndrome) en beroertes zijn bij de ‘oudere patiënt’ chronische complicaties zoals nierfalen, pulmonale hypertensie, retinopathie, galsteenlijden en ijzerstapeling belangrijke determinanten van SCZ gerelateerde morbiditeit en mortaliteit.

In het eerste deel van het proefschrift worden klinische aspecten van SCZ bestudeerd. In hoofdstuk 1 wordt een algemeen overzicht gegeven over SCZ, gevolgd door de huidige inzichten in de pathofysiologie van de ziekte.

In het tweede hoofdstuk wordt de prevalentie van de verschillende ziekte gerelateerde complicaties in een representatief cohort patiënten met SCZ in Nederland beschreven. Geconcludeerd wordt dat er onafhankelijk van de frequentie van vaso-occlusieve crises verschillende vormen van orgaanschade voorkomen. Dit onderstrept het concept dat frequentie van vaso-occlusieve crises, hoewel het historisch als belangrijke determinant van de ernst van de ziekte beschouwd wordt, niet te gebruiken is als maat van ernst van de ziekte in de dagelijkse praktijk. Aangezien sommige vormen van orgaanschade zich in aanvang ontwikkelen zonder duidelijke symptomen is het aangewezen om alle patiënten met SCZ te evalueren op de aanwezigheid van orgaanschade, om potentiële therapeutische interventies zo vroeg mogelijk te kunnen uitvoeren.

In het derde hoofdstuk wordt continue infusie van morfine vergeleken met patiënt gecontroleerde analgesie (patient controlled analgesia (PCA)) voor de behandeling van pijn tijdens vaso-occlusieve crisis door middel van een gerandomiseerd patiëntgebonden onderzoek. Het gebruik van PCA bij patiënten met SCZ tijdens vaso-occlusieve crisis leidt tot een significante vermindering van het morfinegebruik en tot verminderd van morfine gerelateerde bijwerkingen met een vergelijkbare pijnstilling, pijnbeleving en kwaliteit van leven. Bij de behandeling van vaso-occlusive crisis zou PCA dan ook verkozen moeten worden boven een behandeling met continue toediening van morfine. Toekomstige studies zullen echter moeten aantonen of PCA ook beter is dan morfine bolus injecties. De hierboven beschreven bevindingen zijn inmiddels opgenomen in de recent gepubliceerde richtlijnen voor de behandeling van SCZ in Nederland en de Nederlands Antillen.

Een objectieve methode om een vaso-occlusieve crisis te diagnosticeren of de uitgebreidheid van de microcirculatoire obstructie vast te leggen is momenteel niet voorhanden. Een dergelijke method
zou echter van grote waarde zijn voor de behandeling van SCZ; zowel bij het diagnosticeren als ook bij het evalueren van therapeutische interventies. In de studie die beschreven is in het vierde hoofdstuk werd de sublinguale microcirculatie gevisualiseerd en werd de microvasculaire bloedstroomsnellen gegeven bij gezonde controles en patiënten met SCZ tijdens vaso-occlusieve crisis en tijdens ‘steady state’ (de klinisch asymptomatische fase van SCZ) met een nieuwe techniek genaamd Side-stream Dark Field (SDF) imaging.5 Er konden geen verschillen in sublinguale bloedstroomsnellenheid tussen gezonde vrijwilligers en patiënten zonder dan wel met vaso-occlusieve crisis worden aangetoond. De sublinguale bloedstroomsnellenheid was niet veranderd in patiënten met SCZ en derhalve geen bruikbare methode is om het vaso-occlusieve proces te monitoren.

Het tweede deel van dit proefschrift richt zich op pathofysiologische aspecten van SCZ. SCZ wordt gekarakteriseerd door verhoogde stollingsneiging dat wordt veroorzaakt door meerderde factoren, zoals chronische hemolyse en circulerende micropartikels.6 Er is echter geen consensus over wat de cellulaire origine is van deze micropartikels en het exacte mechanisme waarmee zij zouden kunnen bijdragen aan stollingsactivatie in SCZ. In hoofdstuk vijf wordt de cellulaire afkomst van de in SCZ patiënten circulerende micropartikels bepaald tijdens vaso-occlusieve crisis en gedurende steady state. Tevens wordt de relatie met stollingsactivatie, fibrinolyse en endothelactivatie bestudeerd. Het grootste deel van de micropartikels bleek afkomstig van rode bloedcellen en bloedplaatjes. Het totaal aantal micropartikels verschilde niet tussen vaso-occlusieve crisis of steady state. Parameters van in vivo stollingsactivatie, fibrinolyse en endothelactivatie bleken gecorreleerd aan het aantal micropartikels afkomstig van rode bloedcellen. Uit trombinegeneratieproeven bleek de trombinegeneratie bijna met de helft verminderd te kunnen worden door het toevoegen van antistoffen tegen humaan factor XI. Deze experimenten suggereren een belangrijke rol voor factor XI-afhankelijke trombine generatie door micropartikels afkomstig van rode bloedcellen bij SCZ. De waarde van micropartikels als surrogaat parameter bij het evalueren van therapeutische interventies die zich richten op het proces van het ‘sikkelen’ van rode bloedcellen (waarbij de micropartikels vrij komen) behoeft nadere studie.

Een andere factor die mogelijk van belang is bij de pathofysiologie van stollingsactivatie en microcirculatie is beschadiging van de endotheliale glyocalyx. Nieuwe inzichten op het gebied van de endothelbiologie hebben duidelijk gemaakt dat het endothel normaaliter afgeschermd is van circulerende bloedcellen en eiwitten door de glyocalyx; een celvrij waterig netwerk van membraan gebonden eiwitten vetten en suikers aan de binnenkant van de vaatwand.7 De laatste jaren is duidelijk geworden dat endothelactivatie en disfunctie een centrale rol spelen in het proces van vaso-occlusie bij SCZ.8

In hoofdstuk zes wordt aangetoond dat patiënten met SCZ gekarakteriseerd worden door een afgenomen glyocalyxvolume. Gehypothetiseerd kan worden of de glyocalyx-volumevermindering
een nieuwe factor zou kunnen zijn in de pathofysiologie van SCZ gezien de potentiële bijdrage van een beschadigde glycoalyx aan vaso-occlusie bij SCZ.

Hoewel de meeste onderzoeken naar nieuwe behandelingen voor SCZ het effect van de behandeling gemeten hebben aan de hand van de frequentie van vaso-occlusieve crises, blijkt dit een slechte maat voor ziekteactiviteit te zijn. Klinische studies met als eindpunt orgaanschade of mortaliteit zijn echter moeilijk haalbaar en daarom zijn alternatieve objectieve parameters van ziekteactiviteit hard nodig. In dit kader zou monitoren van het glycoalyxvolume een mogelijke nieuwe parameter kunnen zijn om het effect van nieuwe behandelingen in SCZ te evalueren.

In het derde deel van het proefschrift wordt SCZ-gerelateerde pulmonale hypertensie (PHT) bestudeerd. Deze complicatie van SCZ komt veel voor bij deze patiëntengroep en wordt bovendien geassocieerd met hoge mortaliteit. De pathofysiologie van SCZ-gerelateerde PHT is voor een groot deel onbekend. Een belangrijke rol wordt echter toegeschreven aan de door hemolyse veroorzaakte verminderde biologische beschikbaarheid van stikstofmonoxide (NO).9 Het is van groot belang om behandelbare oorzaken of bijdragende factoren van PHT bij SCZ te ontdekken. Voorbeelden van bekende oorzaken of bijdragende factoren van PHT in het algemeen zijn: (in situ) pulmonaal arterie trombose, chronisch obstructief longlijden (COPD), interstitiële longziekten en hypoxie geïnduceerde vasoconstrictie.10-12 Al deze factoren zijn ook beschreven in SCZ, maar hun relatie met SCZ-gerelateerde PHT is onduidelijk. Een verhoogde plasmaconcentratie van asymmetrisch dimethylarginine (ADMA), een endogene remmer van NO-aanmaak, is recent gerelateerd aan ernst en prognose van PHT bij idiopathisch en chronisch trombo-embolische PHT,13 en blijkt verhoogd te zijn in SCZ.14 Uit een prospectief geanalyseerde groep patiënten met voornamelijk milde SCZ-gerelateerde PHT kwamen de volgende bevindingen:

- SCZ-gerelateerde PHT:
  - komt veel voor bij patiënten met SCZ in Nederland (hoofdstuk 2);
  - is niet gerelateerd aan de frequentie van vaso-occlusieve crises (hoofdstuk 2);
  - wordt niet veroorzaakt door obstructie van groot tot middelgrote pulmonale arteriën (hoofdstuk 7);
  - is niet geassocieerd met verhoogde stollingsneiging (hoofdstuk 8);
  - is niet geassocieerd met schade aan longparenchym of abnormale longfunctie testen (hoofdstuk 9);
  - is geassocieerd met plasma asymmetrisch dimethylarginine (ADMA) concentraties (hoofdstuk 10).
Hoewel verschillende studies aangetoond hebben dat SCZ-gerelateerde PHT een risicofactor voor vroegtijdig overlijden is, blijft het onduidelijk of PHT zelf de directe oorzaak is van het overlijden of dat PHT beschouwd moet worden als uiting van algemene ernst van de ziekte. Omdat PHT in het algemeen wordt geassocieerd met een verminderde inspanningstolerantie werd de symptoom gelimiteerde cardiopulmonale inspanningstesten (symptom limited cardiopulmonary exercise testing (CPET)) uitgevoerd bij patiënten met en zonder PHT. Slechts bij één patiënt kon de verminderde inspanningstolerantie verklaard worden door pulmonale vasculaire beperking (d.w.z. PHT). Het merendeel van de patiënten had een verminderde inspanningstolerantie door andere oorzaken, zoals anemie, verminderde cardiovasculaire functie, verminderde functie van bewegingsapparaat, algemeen verminderde longfunctie of door te weinig inzet. Deze bevindingen zaaien op zijn minst twijfel over de assumptie dat de PHT zelf direct verantwoordelijk is voor de hoge mortaliteit. De observaties dat de pulmonale arteriedruk sterk stijgt tijdens SCZ-gerelateerde complicaties (zoals het acute chest syndroom en de vaso-occlusieve crisis), er plotselinge dood geraapporteerd is bij patiënten met PHT tijdens deze complicaties en er ook milde PHT geassocieerd is met vroegtijdig overlijden, maken een oorzakelijk verband tussen PHT en verhoogde mortaliteit bij SCZ waarschijnlijk.\textsuperscript{15}

De relatie tussen ADMA en PHT bij SCZ onderstreep de belangrijke rol die verminderde NO-beschikbaarheid speelt bij de pathofysiologie hiervan. De mogelijkheid om ADMA-concentraties te gebruiken om het risico op het ontwikkelen van PHT (en andere complicaties van SCZ) in te schatten zijn inmiddels in uitvoering. Vanuit een praktisch oogpunt zou screening op PHT bij SCZ-patiënten alleen moeten gebeuren middels trans-thoracale echografie. Eventueel aanvullend onderzoek, zoals in het algemeen geadviseerd bij PHT, is bij patiënten met milde SCZ-gerelateerde PHT van weinig toegevoegde waarde.
Reference List

Dankwoord
Dankwoord

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Curriculum Vitae
Curriculum Vitae

Ward van Beers (born May 7, 1979) studied medicine at the University of Leiden. During his study he was member of the student council of the medical school, chairman of the medical students association and editor of the students edition of the Dutch Journal of Medicine (NTvG-S). After an internship in Nepal in 2003, he and four fellow students founded the Medicine for Acute Lymphocytic Leukemia foundation. This foundation supports treatment of children with leukemia in Nepal. In 2007 he returned to Nepal to deliver a flowcytometer (Facscan) to the Kanti Childrens Hospital in Kathmandu and train local pediatric hemato-oncologists. In 2004 he started his PhD-course at the department of hematology, at the Academic Medical Center in Amsterdam, under supervision of dr.B.J.Biemond. In 2007 he started his training as a resident at the department of internal medicine in the West Fries Gasthuis.

Figure. Training of pediatric hemato-oncologists in flow cytometry in Nepal