ADMA, Angiogenesis and Clinical Complications in Sickle Cell Disease

Landburg, Precious Pearl

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Stellingen bij het proefschrift: AD MA, Angiogenesis and Clinical Complications in Sickle Cell Disease

1. De verschillen in serumconcentraties van angiogenese gerelateerde eiwitten tussen sikkelcelpatiënten en gezonde controles suggereren een rol van angiogenese in de pathofysiologie van sikkelcelziekte. ~ *dit proefschrift*
2. De hoeveelheid circulerende endotheelcellen lijkt gerelateerd aan de mate van orgaanschade bij sikkelcelpatiënten maar is niet gerelateerd aan serum- en plasma markers van endotheelactivatie. ~ *dit proefschrift*
3. Serumconcentraties van SDF-1 zijn in de klinisch asymptomatische fase van sikkelcelpatiënten hoger dan bij mensen met ernstige ziekte zoals sepsis en zijn gerelateerd aan de aanwezigheid van pulmonale hypertensie bij sikkelcelziekte. ~ *dit proefschrift*
4. Om sikkelcelpatiënten beter te kunnen behandelen zijn objectieve markers nodig om de ontwikkeling van orgaanschade te monitoren. ~ *dit proefschrift*
5. Asymmetrische dimethylarginine speelt geen belangrijke rol tijdens de vaso-occlusieve crise, maar lijkt een rol te spelen in de ontwikkeling van pulmonale hypertensie bij sikkelcelziekte. ~ *dit proefschrift*
6. Serumspiegels van angiogenese factoren lijken niet gerelateerd te zijn aan de aan- of afwezigheid van aan sikkelcelziekte gerelateerde complicaties. ~ *dit proefschrift*
7. Statistics are like bikinis. What they reveal is suggestive, but what they conceal is vital. ~ *Aaron Levenstein*
8. It doesn't mean something is useless just because it doesn't do what you planned it to do. ~ *Thomas Alva Edison*
9. Watch the little things; a small leak will sink a great ship. ~ *Benjamin Franklin*
10. Hoe hoger een punt op de politieke agenda, des te meer tijd en middelen hiervoor uitgetrokken worden. Afrikaanse of aan Afrika gerelateerde problematiek staat überhaupt niet op de agenda.

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Ignorance is the night of mind a night without moon or stars

"Confucius"

In nagedachtenis van mijn oma's Esseliene en Hariette
*Because you truly were phenomenal women**

**Maya Angelou*

Paranimfen: Cheryl Engels
Miranda Saphtu-Suripatty

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Contents

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Introduction to and outline of this thesis

P.P. Landburg, J.B. Schnog and A.J. Duits

INTRODUCTION

Background

Sickle cell disease (SCD) is an autosomal recessively inherited hemoglobinopathy caused by a single amino acid substitution of valine for glutamic acid (GAG→GTG) in the sixth codon of the β-globin gene leading to the synthesis of sickle hemoglobin (HbS).[1,2] HbS chains form polymers in the deoxygenated state, leading to the formation of characteristic sickle cells.

The HbSS genotype also known as sickle cell anemia is the most severe form which is characterized by the homozygous expression of the defective gene that codes for HbS. There are also several heterozygote forms known such as HbSC, HbSD and HbSE which are less severe as the homozygote HbSS form. The geographic origin of HbS lies in regions of the world where malaria is endemic (i.e., Africa, South Asia, the Middle East, and around the Mediterranean) as result of the fact that the heterozygote condition (HbAS or sickle cell trait) confers relative resistance to falciparum malaria and thus confers a survival advantage.[3] As a result of slavery and migration the disease is nowadays present throughout the world with an estimated prevalence of sickle cell trait 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.[4] In Curaçao, a small island in the Caribbean with 140.000 inhabitants, the prevalence is estimated to be 5.0% for HbAS with a prevalence of about 0.07% HbSS and 0.18% HbSC patients.[5]

SCD is an important hemoglobinopathy characterized by recurrent micro-vascular vaso-occlusion, chronic intravascular hemolysis and reduced life expectancy.[6,7]

PATHOPHYSIOLOGY

The mechanisms responsible for the development of SCD-related complications are focus of intense research. Many mechanisms contribute to the complex pathophysiology of SCD, with dysfunction of the vascular endothelium being considered a factor of major importance. A central role in the pathophysiology of SCD is attributed to hemolysis and vaso-occlusion.

Hemolysis

In SCD hemolysis is driven by HbS polymerization, in such a way that recurrent deoxygenation and reoxygenation of erythrocytes result in cell membrane damage. Hemolytic anemia varies in intensity among the different genotypes of SCD. It is most severe in the homozygote genotype, less severe in double heterozygote genotypes and also less severe with co-inheritance of α-thalassemia deletion. Hemolysis contributes to the development of vasculopathy which is characterized by pulmonary and systemic hypertension, endothelial dysfunction and proliferative changes in the intima and smooth muscle of blood vessels.[8-10] Several complications such as priapism, cholelithiasis, leg ulcers and pulmonary hypertension (PHT) have been shown to be associated with increased rates of hemolysis resulting in a reduced nitric oxide (NO) bioavailability.[8,11] The hemolytic rate does not seem to be associated with the frequency of vaso-occlusive events. Due to the fact that patients with low hemoglobin concentrations and high hemolytic rates are more likely to develop vasculopathy than are those with higher hemoglobin concentrations, whom also seem more prone to episodes of acute painful crisis and possibly acute chest syndrome (ACS), some have proposed that SCD patients seem to form distinct subphenotypes driven by hemolysis on one and vaso-occlusion on the other hand.[12,13]

Vaso-occlusion

Polymerization of erythrocyte hemoglobin has been demonstrated to be responsible for the characteristic sickle shape and is thought to play an important role in the development of vaso-occlusion. Several decades ago vaso-occlusion was understood as a transient log-jam of sickle shaped erythrocytes within the small vessels impeding local blood flow which resulted in painful crisis until spontaneous resolution of vaso-occlusion. Hereafter the critical role of the endothelial cells (ECs), adhesion molecules, leukocytes and platelets in the pathophysiology of vaso-occlusion became increasingly clear. Cell adhesion, of both red and white blood cells, alter the hemodynamics of the microvasculature leading to a reduced flow rate promoting HbS polymerization, hence leading to occlusion of the microvasculature due to trapping of cells. Leukocytes contribute directly to the vaso-occlusive process and elevated leukocyte counts are

considered a risk factor for stroke, ACS and early SCD-related death.[7,14-17] Many factors and processes have been shown to be involved in leading to endothelial activation, damage and dysfunction.[6]

Painful crisis is most often the resultant of vaso-occlusion and due to ischemia-reperfusion injury multiple organ systems in SCD patients are affected leading to complications like osteonecrosis and ACS representing an important source of SCD-associated morbidity and mortality.

ENDOTHELIAL DYSFUNCTION

Hemolysis and vaso-occlusion lead to endothelial activation and dysfunction therefore inducing a chronic inflammatory state which is propagated by elevated blood levels of inflammatory cytokines, adhesion molecules and angiogenic factors.[18,19] Here NO bioavailability, angiogenesis and vasoregulation, which is the regulation of vascular homeostasis by maintaining the balance between endothelial derived vasodilatory and vasoconstrictive factors, will be discussed in more detail.

Nitric oxide bioavailability

Sickling and unsickling of erythrocytes results in cell membrane damage leading to the release of hemoglobin. NO reacts with plasma hemoglobin producing methemoglobin and bio-inactive nitrate or iron-nitrosyl-hemoglobin resulting in irreversible NO scavenging pathways. A state of reduced endothelial NO bioavailability in SCD contributes to vasoconstriction and impairs downstream homeostatic vascular functions of NO like transcriptional repression of the cell adhesion molecules such as vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, P-selectin and E-selectin. Due to hemolysis erythrocytes also release arginase. Arginase depletes plasma arginine by increasing the production of ornithine at the expense of citrulline production, thus further compromising NO bioavailability.[20] Hence, the normal balance of vasoconstriction to vasodilation is therefore skewed toward vasoconstriction, endothelial activation and proliferation.[21]

Vasoregulation

The endothelium regulates vascular homeostasis by regulating vasomotor tone, blood flow, growth of vascular smooth muscle cells and local inflammation.[22] In addition to their constitutive production of the major vasodilator, NO, the ECs also produce other vasodilators such as prostacyclin, as well as vasoconstrictors including endothelin-1, angiotensin II and prostaglandins.[23] In SCD, the finely-tuned production of EC vasoregulators is imbalanced. As mentioned earlier it is recognized that SCD is characterized by a reduced bioavailability of NO contributing to an increase in vasoconstriction that is thought to play a role in occurrence of complications such as PHT,[24] as well as vaso-occlusion. Asymmetric dimethylarginine (ADMA), which is an endogenous inhibitor of NO synthase, is thought to contribute to the reduced NO availability in SCD next to the increased arginase level and the increased amount of reactive oxygen species.[25] Reinforcing the decrease in endothelial vasodilatory NO, upregulation of vasoconstrictive factor production in the endothelium has been reported in SCD. Elevated levels of plasma endothelin-1, a potent long-acting mediator of vasoconstriction that is produced by endothelial and vascular smooth muscle cells in response to inflammatory stimuli, hypoxia and shear stress, have been found during steady state in SCD and appear to increase further during crisis.[26-28]

ANGIOGENESIS

Angiogenesis is the result of highly orchestrated series of molecular and cellular events, resulting in the migration, proliferation and differentiation of ECs into newly formed capillaries which can subsequently develop into more mature vessels. The microvascular vessel tone and structure is regulated by several factors, of which NO is a key regulator. Exposure of ECs to low oxygen tension results in a profound decrease in endothelial NO synthase transcription corresponding with a fall in endothelial NO synthase protein level which leads to a co-ordinate impairment of production of NO.[29] The importance of NO to the physiology of vasomotor and endothelial activity is exemplified during chronic shortage leading to human cardiovascular diseases associated to endothelial dysfunction.[30-32] Chronic decreased NO bioavailability

contributes to endothelial activation and vascular wall remodeling due to the ability of modulating the EC production and release of growth factors.[33-36] Angiogenic factors and vasoactive peptides, such as erythropoietin, platelet derived growth factor-β and endothelin-1, are known to be released from a variety of cells due to prolonged or severe hypoxia.

The vascular endothelium in healthy adults is normally quiescent, yet after endothelial injury circulating (ECs) are mobilized in order to take part in endothelial neovascularization. Endothelial progenitor cells (EPCs) are CD34+ endothelial hematopoietic cells which are mobilized from the bone marrow in order to contribute to re-endothelialization.[37,38] Increased growth factor release such as stromal cell-derived factor-1 is thought to be responsible for EPC mobilization by enhancing the homing of EPCs and augmenting neovascularization in ischemic tissue. Moreover increased circulating EPC counts are found in chronic inflammatory diseases such as coronary artery disease.[39]

Endothelial activation and release of angiogenic factors play a role in the development of mature and stable vessels and also in the development of PHT, cancer and other vascular diseases.[40,41] In SCD increased levels of adhesion molecule expression (ICAM-1, VCAM-1, P-selectin), inflammatory and anti-inflammatory cytokines (IL-6, IL-8, TNF-α) have been described to contribute to the development of organ injury. Many key angiogenic proteins have been found to differ between sickle cell patients and healthy controls.[6,42-44] The role of circulating angiogenic factors in the development of SCD-related complications is still to be elucidated. Due to a specific level profile of the angiogenic factors SCD patients have been postulated to be in a pro-angiogenic state of which the endothelial activation in combination with vaso-constriction could contribute to unbalanced angiogenesis leading to organ dysfunction.[11,19,45,46] As of yet mainly the role of angiogenic factors in the pathophysiology of SCD-related PHT is being studied. Herein altered levels of placenta-like growth factor (PlGF), vascular endothelial growth factor (VEGF) and platelet derived growth factor levels, among others, have been suggested to be of importance in both children and adults.[47] Angiogenesis has also proven roles of importance in other complications that occur in SCD, such as retinopathy, *Moyamoya* syndrome and stroke. However, studies seeking

to find relationships between serum/plasma concentrations of angiogenic proteins and the presence of SCD-related complications have been inconsistent in results.

CLINICAL MANIFESTATIONS IN SCD

Chronic hemolysis and recurrent vaso-occlusion are responsible for the clinical features of SCD due to progressive development of disabling organ damage such as PHT, ACS, stroke, osteonecrosis and renal failure.[6]

Until birth the β-globin genes are inactive and the γ-globin genes are responsible for the production of fetal hemoglobin (HbF), hence a neonate is asymptomatic since a major part of the total hemoglobin is HbF. As of 6 months the HbF level decreases and symptoms start to appear such as avascular necrosis resulting in shortening of a digit, splenic or hepatic sequestration, in which pooling of erythrocytes in a rapidly enlarging spleen or liver leads to acute aggravation of anemia leading to circulatory collapse. In all ages but mainly during childhood recurrent infarction of the spleen primarily leads to dysfunction and eventually, especially in HbSS, to functional asplenism. Patients with asplenism show an increased susceptibility to various infections, mainly with encapsulated bacteria such as pneumococci.[48-50] Parvovirus B19 infection can result in acute worsening of anemia due to a total arrest of erythropoiesis by invading the red cell precursor. The increased life expectancy in SCD leads to an increment in chronic complications in adults affecting internal organs.[51]

Acute painful crisis

The most frequently seen complication and the most common cause of hospitalization is the acute painful crisis which can be triggered by infection, heavy physical exertion, high altitude and dehydration but which can also develop spontaneously.[7,52,53] A painful crisis is generally the resultant of acute vaso-occlusion which in patients with SCD is caused by an increase of ischemic tissue injury resulting from the occlusion of microvascular beds by sickled erythrocytes. The pain most commonly involves the back, legs, knees, arms, chest and abdomen.

An acute abdominal pain crisis can mimic an intra-abdominal process such as cholecystitis or appendicitis resulting in surgery. In the majority of cases during surgery

no specific cause is identified and spontaneous resolution occurs. The frequency, severity, and duration of these crises vary considerably.

Pulmonary hypertension

In SCD and namely in homozygous sickle cell anemia, pulmonary complications such as PHT have emerged as a significant independent risk factor of early death yet the mortality rate and true prevalence have recently been challenged.[8,13,54-56] Even more a recent study showed that the prevalence of PHT in SCD detected on echocardiographic screening has probably been overestimated.[56] In former studies the prevalence of PHT in SCD when using Doppler echocardiography, with PHT defined as tricuspid regurgitant jet velocity of ≥2.5 m/s, is estimated to be 30% with a mortality rate of 5.3%.[8] When using right heart catheterization, which in international guidelines is recommended as standard of care, the prevalence has recently been shown to be 6% with a mortality rate of 2%.[56]

The pathogenesis of PHT in SCD is likely multifactorial and intravascular hemolysis, impaired NO bioavailability, altered endothelial function, continuous vaso-occlusion in the micro-vasculature and ischemia-reperfusion injury are thought to play a role. Chronic lung injury may lead to fibrotic pulmonary parenchymal damage, hypoxia and a pulmonary vasculopathy. Nevertheless ACS, which is a potential cause of chronic lung disease and pulmonary fibrosis, is not associated with PHT.[57]

Acute chest syndrome

Next to PHT ACS is a very unique SCD-related complication which is the second most common cause of hospitalization and premature death. ACS also is the leading cause of admission to an intensive care unit in this patient population.[58] The incidence of ACS is highest in children 2–4 years of age and, while gradually declining with age, remains common in adults.[59] Severity varies, but 13% of patients require mechanical ventilation and 3% die.[58] ACS can be defined as a presence of a new infiltrate not due to atelectasis, involving at least one complete lung segment, chest pain, fever and tachypnea. Current understanding of the pathophysiology is limited but ACS may be caused by airway infection, fat emboli or pulmonary infarction yet most cases develop during vaso-occlusive crisis without a clear explanation.[58] Also decreased NO

bioavailability leading to vasoconstriction in the pulmonary vasculature and hemolysis are thought to be mechanisms of importance in the development of ACS.[60]

TREATMENT

SCD is a devastating disease with acute and chronic manifestations. These complications may result in hospitalization and poor quality of life. Since the pathophysiology is not completely understood treatment is mainly symptomatic and preventive. The care of patients with SCD has undergone important advances in recent years. In western countries institution of newborn screening programs, provision of pneumococcal vaccination and prophylaxis with penicillin in childhood is administered and effects of efficacious drugs, such as hydroxycarbamide (also known as hydroxyurea) are better understood. New therapies and drugs such as gene therapy, administration of NO and L-glutamine, are currently being examined as possible alternatives for pharmacotherapy. They are at different stages of basic and clinical investigation. The concepts of individualized therapy and combination therapy have been further explored in more recent years, also offering promise to improve the care of patients with SCD.[61]

Stem cell transplantation is the only curative therapy with an estimated mortality risk of 7-15% yet due to lack of suitable matched donors and the chronic use of immunosuppressive drugs with serious side effects the treatment is rarely used.[62,63]

Painful crisis is the most frequently seen complication which patients often can manage at home by using pain medication like acetaminophen, non steroidal anti-inflammatory drugs and codeine.[64,65] When hospital administration is necessary for treatment of a painful crisis an underlying cause, such as an infection, should be sought and intravenous opioids, oxygen and fluids administered.

In treating ACS the primary goal is to correct underlying factors that contribute to deoxygenation and reduction of the amount of HbS, ischemia and preventing (further) injury to the lung. In 30% of cases an infection was determined, hence once ACS has been diagnosed antibiotics are usually given although this does not seem to shorten the clinical course.[66] Hydroxycarbamide is known to decrease the rate of ACS and blood or exchange transfusion has been shown to produce improvement.[58]

For SCD-related PHT there is no proven treatment. Regular blood transfusion

and anticoagulation have been suggested.[13,67] Prostacyclin analogues, endothelin-1 receptor antagonist (Bosentan), phosphodiesterase-5 inhibitors (Sildenafil) and calcium channel blockers are being evaluated in the treatment of PHT in SCD.

THESIS OUTLINE

SCD is a complex and severe systemic disease in which hemolysis and vaso-occlusion are both known to result in endothelial activation leading to impaired angiogenesis. Angiogenesis is likely to be of importance in SCD-related (patho)physiology.[6] Nevertheless the factors important for the angiogenic response are not known and the importance of circulating angiogenic factors in the development of SCD-related complications remains to be elucidated.

Therefore the aim of this present thesis is:

1. To explore the role of angiogenesis in SCD-related complications.
2. To study the role of specific factors that decrease NO bioavailability in SCD

In this thesis chapter 2 and 3 address results of studies on pro- and anti-angiogenic factors, their possible role in maintaining the pro-angiogenic state as well as their relation to the presence of disease-related complications. In chapter 4 and 5 the role of circulating ECs is studied in SCD. Lastly the importance of ADMA, the endogenous inhibitor of NO-synthase, in the pathophysiology of SCD is set out in chapter 6, 7 and 8.

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2

Increased Serum Levels of Anti-Angiogenic Factors Soluble Fms-Like Tyrosine Kinase and Soluble Endoglin in Sickle Cell Disease

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ABSTRACT

The anti-angiogenic factors soluble Fms-like tyrosine kinase (sFlt)-1 and soluble endoglin (sEng) have been shown to be of importance in angiogenesis by sequestering and inhibiting vascular endothelial growth factor (VEGF), placenta-like growth factor (PlGF) and transforming growth factor-β1 signaling. Given the potential role of angiogenesis in the pathophysiology of sickle cell disease (SCD)-related complications, serum levels of sFlt-1 and sEng were determined in SCD patients and controls. Both sFlt-1 (p = 0.002) and sEng (p = 0.004) were elevated in patients during clinically asymptomatic SCD with no further increment during painful crisis. These data suggest that sFlt-1 and sEng may be important in the regulation of angiogenesis in SCD.

Introduction

Next to vaso-occlusion-induced ischemic organ damage, the formation of abnormal blood vessels (e.g. sickle retinopathy or *Moyamoya* disease) and the process of vascular remodeling (pulmonary hypertension) contribute significantly to sickle cell disease (SCD)-related morbidity.[1] Such pathologic processes are, at least in part, driven by hypoxia and inflammation-induced angiogenesis. Angiogenesis is a well-orchestrated process determined by the relative levels of pro- and anti-angiogenic proteins, as well as target cell receptor expression.[2] We have recently shown elevated angiopoietin (Ang)-2 levels in relation to Ang-1 and vascular endothelial growth factor (VEGF) levels in patients with SCD, suggesting a pro-angiogenic state.[3]

Several studies have shown the importance of the anti-angiogenic factors soluble Fms-like tyrosine kinase (sFlt-1) and soluble endoglin (sEng) in disease states associated with abnormal angiogenesis such as preeclampsia.[4] sFlt-1 is a soluble form of VEGF receptor-1, which binds to and sequesters circulating free VEGF and free placenta-like growth factor (PlGF), thereby neutralizing their pro-angiogenic effects.[4] sEng is the soluble form of the transforming growth factor (TGF)-β co-receptor Eng and is known to bind and inhibit TGF-β1 signaling.[4] TGF-β1 is also a pro-angiogenic protein, and the TGF-β pathway has been associated with several major SCD-related complications such as pulmonary hypertension,[5] leg ulcers,[6] stroke and priapism.[7,8] Given the new insights into angiogenesis as a factor of importance in the pathophysiology of SCD, we set out to characterize serum profiles of sFlt-1 in relation to VEGF and PlGF levels, as well as sEng levels in SCD patients during the clinically asymptomatic state and during acute vaso-occlusive crises.

Patients and Methods

Patients

Fourteen HbSS and 9 HbSC patients were consecutively included in this study. Blood samples were drawn in clinically asymptomatic state and during a vaso-occlusive crisis (defined as an episode of acute pain in the abdomen and/or extremities not

otherwise explained) from the same patients. None of the patients were on any kind of treatment except for folic acid. Thirty-four HbAA blood donors served as healthy controls. Written informed consent was obtained from patients and controls. This study was approved by the local ethical review board and conducted in agreement with the Helsinki Declaration of 2000.

Reagents

Blood was collected and serum prepared according to standard procedures and frozen at –80°C. Serum sFlt-1, sEng, VEGF and PlGF levels were measured by enzyme-linked immunosorbent assay (R&D Diagnostics, Minneapolis, Minn., USA) according to the manufacturer's instructions.

Data Analysis and Statistics

For multiple group comparisons of continuous variables, the Kruskal-Wallis test was employed. For paired sample analysis (clinically asymptomatic state compared to vaso-occlusive events), the Wilcoxon signed-rank test was used. p Values ≤ 0.05 were considered statistically significant. The Statistical Package for Social Sciences (version 14.0, SPSS ^{Inc}, Chicago, Ill., USA) was used.

RESULTS

Serum levels of sFlt-1 and sEng were elevated in the asymptomatic state of both HbSS and HbSC patients compared with healthy controls (Table 1), but did not increase further at presentation during a painful crisis. In both the asymptomatic and vaso-occlusive phase, VEGF and PlGF serum levels were comparable to controls (Table 1), even though VEGF levels increased in a significant number of HbSC patients during vaso-occlusive crises ($p = 0.02$, data not shown).

Table 1 | Anti- and pro-angiogenic factors in clinically asymptomatic patients and healthy controls

	HbSS (n = 14)	HbSC (n = 9)	Control (n = 34)	*p*-value
sEng, ng/ml	6.3 (5.4 - 8.4)	6.1 (3.9 -7.1)	4.1 (3.4 -5.1)	<0.01
sFlt-1, pg/ml	215 (130 - 314)	182 (116 - 294)	21 (0 - 154)	<0.01
VEGF, pg/ml	344 (176 - 678)	146 (81 -492)	261 (143 - 453)	0.51
PlGF, pg/ml	11.3 (7.6 - 14.2)	8.9 (4.2 -12.7)	10.7 (7.8 - 13.8)	0.63

Data are shown as medians with interquartile ranges. The Kruskal-Wallis test was employed for between group analysis.

Discussion

The process of angiogenesis may play a role in determining the rate of tissue regeneration and in the development of specific SCD-related complications such as sickle retinopathy, *Moyamoya* disease and pulmonary hypertension. In SCD, enhanced Ang-2 levels in the presence of unaltered VEGF levels suggest a pro-angiogenic state.[3] Circulating levels of the anti-angiogenic factors sFlt-1 and sEng were determined in SCD patients since they emerged as important inhibitors of angiogenesis. In this study, serum sFlt-1 and sEng levels were statistically significantly increased in clinically asymptomatic SCD patients compared with healthy controls, with no further increments during an acute vaso-occlusive crisis. sFlt-1 has been implicated in pathological angiogenesis and inflammation by sequestering circulating free VEGF and PlGF.[4] In preeclampsia, elevated sFlt-1 levels contribute to endothelial dysfunction and the development of renal failure and proteinuria. The sFlt-1 elevations in our patients could be hypoxia driven via induction of hypoxia-induced factor (HIF)-1α following continuous tissue hypoxia secondary to ongoing microvascular occlusion.[9] Neither free VEGF nor free PlGF serum levels were elevated in our patients. VEGF expression is upregulated by pro-inflammatory cytokines and hypoxia,[10] and PlGF expression is induced by erythropoietin (EPO),[11] with VEGF and PlGF targeting the same receptors.[10] Recently, it has been demonstrated that in preeclampsia free VEGF (and PlGF) levels decreased probably due to sequestration by elevated sFlt-1 levels despite hypoxia-induced increments in total circulating VEGF levels,[4] possibly

indicating that the lack of increments in free VEGF and PlGF during steady state in our patients also results from sFlt-1 sequestration. The potent anti-angiogenic role of sEng, a potential N-terminal cleavage product of the full-length TGF-β co-receptor Eng, has recently been described.[12] sEng is known to inhibit activated TGF-β1 binding to its receptor on endothelial cells and its serum levels were significantly elevated in SCD patients during the asymptomatic state.[13] We (and others) could not measure circulating TGF-β1 as it is probably complexed to several inhibiting proteins and unable to bind to its receptors (data not shown).[12] Locally generated active TGF-β1 is known to stimulate endothelial nitric oxide synthetase expression.[12] The elevated sEng levels could, via local inhibition of TGF-β1 signaling, therefore contribute to the recently recognized reduced NO bioavailability in SCD,[14] a factor now appreciated as a central factor in the development of major SCD-related complications.

In evaluating both the role of sFlt-1 and sEng in SCD, consideration should be given to the fact that the increased absolute concentrations are (substantially) lower than those measured during preeclampsia, which could very well reflect the etiological differences of SCD-related angiogenic activity and chronic endothelial dysfunction, and angiogenesis and endothelial dysfunction during this pregnancy-related complication.

During a painful crisis, serum sFlt-1 levels did not further increase, in agreement with an in vitro model of placental hypoxia demonstrating HIF-1α-regulated sFlt-1 expression to be unaffected by intermittent hypoxic stress.[9] Interestingly, Eng expression is also primarily regulated by HIF-1α,[15,16] and the unaltered levels during acute vaso-occlusive crises could be explained by the same mechanism. EPO is an important PlGF inducer and a trend to higher EPO levels was observed in a larger group of SCD patients during painful crises with a significant increment in PlGF levels.[3,11] In this admittedly smaller group of patients, these findings could not be reproduced. During painful crises, VEGF did increase only in HbSC patients (data not shown). Lack of further increments in the measured factors does not exclude potentially important roles in the development of SCD-related complications, since evidence is accumulating that the development of organ damage and dysfunction is often not related to the occurrence of acute vaso-occlusive events.[17,18]

Contrary to our findings, Mohan *et al* did not detect elevated sFlt-1 levels in their study on clinically asymptomatic SCD patients.[19] However, comparison with our data is difficult since the authors do not discern between values for HbSS and HbSC patients (which may be substantially different according to our data). Furthermore, citrated plasma was employed (compared to serum), perhaps indicating a methodological basis for the observed discrepancies.

In conclusion, our data indicate that, during the clinically asymptomatic state, SCD patients are characterized by increased serum levels of the anti-angiogenic factors sFlt-1 and sEng, further supporting the concept that SCD is characterized by an altered angiogenic state. The net effect of sFlt-1 and sEng inhibition of VEGF, PlGF and TGF-β1 on angiogenesis in SCD, as well as the potential of identifying patients at risk for specific complications by monitoring serum sFlt-1 and sEng levels (as was recently demonstrated for preeclampsia) will be the subject of further studies.

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3

Relation Between Serum Concentrations of Angiogenic Factors and the Presence of Sickle Cell Disease-Related Complications

Angiogenic factors in sickle cell disease-related complications

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ABSTRACT

Hypoxia induced angiogenesis plays an important role in the pathophysiology of sickle cell disease (SCD). In order to investigate the association of angiogenic factors with SCD-related complications we measured serum levels of angiopoietin (Ang)-1, Ang-2, vascular endothelial growth factor (VEGF), placenta-like growth factor (PlGF), soluble tunica intima endothelial kinase (sTie)-2, erythropoietin (EPO) and soluble vascular cell adhesion molecule (sVCAM)-1 in a well defined SCD population. We analyzed serum samples of 51 HbSS/HbSβ^{o}-thalassemia and 24 HbSC/HbSβ^{+}-thalassemia patients in absence and presence of SCD-related complications such as pulmonary hypertension, retinopathy and stroke. The angiogenic profiles did not seem to differ between patients with and those without complications.

INTRODUCTION

Endothelial cells (ECs) and angiogenic mediators play important roles in diseases such as cancer, pulmonary hypertension (PHT) and retinopathy.[1,2] Angiogenesis is regulated by cytokines and angiogenic factors such as vascular endothelial growth factor (VEGF), angiopoietin (Ang)-1 and Ang-2. Endothelial receptor soluble tunica intima kinase (sTie)-2 binds angiopoietins with the relative Ang-1/Ang-2 levels in relation to VEGF levels determining the balance between vessel quiescence and angiogenesis.[3] These angiogenic factors favor angiogenesis when in the presence of VEGF Ang-2 is relatively increased to Ang-1, vessel quiescence is maintained when Ang-1 is relatively increased to Ang-2. Placenta-like growth factor (PIGF) is an erythroid cell-derived cytokine belonging to the VEGF family. Next to direct angiogenic effects PIGF release leads to monocyte activation resulting in not only VEGF release,[4] but also increased gene expression of cytokines such as tumor necrosis factor-α, interleukin (IL)-1 and IL-8.[5] In addition to its hematopoietic role, erythropoietin (EPO) is also recognized as an angiogenic growth factor responsible for tumor growth progression.[6]

Abnormal or disrupted angiogenesis likely plays a role of importance in the development of several sickle cell disease (SCD)-related complications. SCD is characterized by continuous EC activation, ongoing (low grade) tissue ischemia and continuously elevated EPO levels due to the hemolytic anemia, all factors known to induce or contribute to angiogenesis. Indeed, angiogenesis has proven roles of importance in several complications that occur in SCD, such as PHT, retinopathy, *Moyamoya* syndrome and stroke. Furthermore, many key angiogenic factors have been found to differ between sickle cell patients and healthy controls.[7-11]

However, studies seeking to find relationships between serum/plasma concentrations of angiogenic proteins and the presence of SCD-related complications have been inconsistent in results.[12] We therefore set out to compare serum levels of several important angiogenic proteins in relation to the presence or absence of disease-related complications in a well defined SCD patient cohort.

PATIENTS AND METHODS

Patients

Forty-six HbSS, 18 HbSC, 6 HbSβ°-thalassemia and 6 HbSβ⁺-thalassemia adult sickle cell patients cared for at the Department of Internal Medicine of the Sint Elisabeth Hospital (Curaçao), the Slotervaart Hospital (Amsterdam, The Netherlands), and the Department of Hematology of the Academic Medical Centre (Amsterdam, The Netherlands) were considered eligible. Written and informed consent were obtained from all patients. This study was approved by the internal review board of the Academic Medical Centre and carried out in accordance with the principles of the Declaration of Helsinki of 1975, as revised in 2000.

SCD-related complications

In the outpatient clinics adult patients are regularly screened for SCD-related manifestations as previously defined and reported:[13] *Micro-albuminuria:* urinary albumin (mg/l) to urinary creatinine (mmol/l) ratio >3.5 (males)/ >2.5 (females) confirmed with 24 hour urine collection with micro-albuminuria >30 mg/24 hours. *PHT:* tricuspid regurgitation jet flow velocity (TRV) ≥2.5 m/s in rest detected by Doppler echocardiography. PHT was considered absent when no or only trace TRV is detected. Mild PHT was defined as TRV 2.5-2.9 m/s and moderate to severe as TRV ≥3.0m/s. *Retinopathy*: presence of at least mild non-proliferative retinopathy. *Cholelithiasis*: presence of gallstones (ultrasound) or previous cholecystectomy because of cholecystolithiasis. Acute chest syndrome (*ACS):* defined as previously described occurring between January 2002 until January 2007.[14] *Symptomatic avascular osteonecrosis (AVN):* 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 ulcer:* chronic ulcer 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. *Painful crises:* the cumulative number of admissions for painful crises (defined as typical musculo-skeletal/abdominal pain not otherwise explained) from January 2002 until January 2007 was used to categorize patients into 2 groups: 0-1 painful crisis per year and 2 or more painful crises per year.

Collection of blood samples

Blood samples were collected during regular outpatient visits from the antecubital vein with a vacutainer® system. Whole blood samples were centrifuged at 1700*g* for 10 min. Serum was collected and aliquots were stored at -70°C until further analysis.

Laboratory determinations

Serum Ang-1, Ang-2, sTie-2, VEGF, PlGF, EPO and soluble vascular cell adhesion molecule (sVCAM)-1 levels were determined by enzyme-linked immunosorbent assay according to manufacturer's procedures (R&D Diagnostics, USA).

Statistical analysis

As previously reported the most severe SCD genotypes (HbSS and HbSβ^o-thalassemia) were grouped together, as were the relatively milder genotypes (HbSC and HbSβ$^+$-thalassemia).[15] Continuous data are presented as medians with their corresponding inter-quartile ranges (IQR). Between group statistical analysis were confined to comparisons where at least four cases were present per group, and organ damage had to be screened for in at least two years prior to study inclusion. Between 2 groups differences were tested with the Mann-Whitney *U* test. For correlation studies the Spearman correlation coefficient (r_s) was calculated. P-values <0.05 were considered statistically significant. Statistical analysis was performed by using Statistical software Package for the Social Science (SPSS), version (SPSS 15.0., SPSS ^{Inc}, Chicago, Ill, USA).

RESULTS

Patient characteristics are shown in Table 1. Apart from retinopathy other complications were rarely seen in the HbSC/HbSβ$^+$-thalassemia group precluding further analysis in this group. Forty-six patients in the HbSS/HbSβ^o-thalassemia group were consecutively screened for PHT with echocardiography as reported elsewhere this group consisted almost exclusively of patients with mild PHT.[16] Only two patients had moderate to severe PHT with TRV levels of 3.0 and 3.5 m/s.

In the HbSS/HbSβ^o-thalassemia group serum sTie-2 concentrations were significantly decreased in patients with retinopathy (28.9pg/mL, 22.9-39.3pg/mL) as opposed to those without (39pg/mL, 28.3-47.3pg/mL) (p=0.035). In patients with PHT

and nephropathy sVCAM-1 levels were higher as opposed to patients without these complications (1053.6ng/mL, 792.1-1200.7ng/mL vs. 1342ng/mL, 937.5-1697.4ng/mL, p=0.049 and 1637.2ng/mL, 1283.6-2241.8ng/mL vs. 1072ng/mL 792.1-1273.6ng/mL, p=0.007, respectively). No other statistically significant differences were detected in measured parameters between patients with and without disease-related complications.

Statistically significant correlations were detected between serum levels of Ang-1 and sTie-2 (r_s=-0.307, p=0.028), Ang-1 and Ang-2 (r_s=-0.287, p=0.041), VEGF and EPO (r_s=0.339, p=0.030). TRV was statistically significantly correlated to serum sVCAM-1 levels (data not shown). No other statistically significant correlations were observed (data not shown).

Discussion

Several studies have shown altered serum levels of angiogenic factors in patients with SCD as opposed to healthy controls, implying a role for (dysregulated) angiogenesis in the pathophysiology of SCD-related complications. In this study, apart from lower serum sTie-2 levels in the HbSS/HbSβ^0-thalassemia group with sickle retinopathy, no relation of angiogenic factor levels to the presence of disease-related complications could be detected.

Retinopathy occurs in greater frequency in double heterozygous forms of SCD which as of yet has not been explained by recent research.[17,18] As was reported for proliferative diabetic retinopathy we measured lower sTie-2 concentrations in HbSS/HbSβ^0-thalassemia patients with retinopathy as opposed to those without.[19] However, this was not the case in HbSC/HbSβ$^+$-thalassemia patients and Mohan *et al* reported no significant increment of sTie-2 plasma levels in a heterogeneous group of sickle cell patients with retinopathy.[20] The different angiogenic profiles of HbSS and HbSC patients might lead to the increased frequency and different pathophysiology of retinopathy in these two sickle genotypes.[8,21,22] The decreased sTie-2 level in HbSS patients is not correlated to Ang-2 or VEGF and decreased level or sustained blockage of the Tie-2 receptor seemed to inhibit neovascularization.[23,24] Hence, one might speculate that the decreased level of sTie-2 in retinopathy and the negative association of Ang-1 to

sTie-2 are more likely to have a protective role in the development of retinopathy in HbSS/HbSβ°-thalassemia patients.

Table 1 | Patient characteristics

	HbSS (N=37)/HbSβ° (N=14)	HbSC (N=18)/HbSβ⁺ (N=6)
N	N=51	N=24
Age (years)	27 (21-47)	30 (23-38)
Male:Female	15:36	13:11
Complications %		
PHT	40	10
Retinopathy	28	53
Nephropathy	18	0
Albuminuria	40	5
Stroke	10	0
Priapism	11	0
Leg ulcers	10	0
≥2 crises/year	13	11
ACS	37	10
Ang-1 (pg/mL)	30655 (19750-46770)	20945 (12755-42405)
Ang-2 (pg/mL)	5525 (3608-7260)	2910 (2017-3633)
Tie-2 (pg/mL)	30 (21-42)	33 (27-41)
VEGF (pg/mL)	485 (273-1204)	280 (224-650)
PlGF (pg/mL)	14 (9-39)	14 (8-43)
EPO (mIU/mL)	71 (35-123)	34 (14-51)
sVCAM-1 (ng/mL)	1118 (847-1357)	719 (601-971)

Demographics and laboratory parameters in SCD patients. Data are presented as medians with their corresponding IQR. PHT= pulmonary hypertension, ACS= acute chest syndrome

Angiopoietins are important angiogenic factors in the development of PHT.[25] However, serum concentrations of Ang-1, Ang-2 and sTie-2 did not differ between patients with and without PHT. VEGF levels were also similar between patients with and without PHT, without a relation to the TRV (as was recently reported in pediatric sickle cell patients), even though elevated serum VEGF levels have been associated with severe PHT.[26] As our patients were almost exclusively characterized by mild PHT we cannot exclude a relation of serum VEGF levels to severe PHT in SCD. Furthermore, several studies have shown a poor correlation between local VEGF expression and serum VEGF levels, indicating that VEGF serum levels might not accurately reflect local VEGF levels to which cells of the pulmonary microvasculature are exposed. Brittain *et al* recently reported an association of plasma PlGF to severe SCD-related PHT.[11] In our patients with mild PHT no relation of serum PlGF to PHT was present. We do not believe that the use of serum instead of plasma has influenced the outcome negatively since higher PlGF levels were measured in serum compared to PlGF levels in plasma in a population of pregnant woman with and without preeclampsia.[27]

We did not find a relation between EPO levels and TRV in our adult SCD population which is in concert with an earlier study.[28] Recently in a pediatric SCD population a relation was found between EPO and TRV assuming a different pathophysiology in the development of PHT then in the adult population.[29]

In a mice study a negative association was seen between Ang-1 and sTie-2 which has been suggested to have a protective role in the development of PHT. In mice this pathway promotes EC survival under stress.[30] Our present data studied in humans with SCD-related mild PHT also showed a negative association between Ang-1 and sTie-2 yet the role of this negative association in the development of PHT in humans still remains to be determined.

Taken together, these data show that serum profiles of angiogenic proteins do not differ between patients with and without specific SCD-related complications. Given the complexity of the angiogenic process and the often not yet defined or poor relation between serum profiles, tissue expression and signal transduction, these data do not imply that angiogenesis does not play a role of importance in

the pathophysiology of SCD. Conceding the relatively small sample size, our data do indicate that disease severity monitoring with serum levels of angiogenic factors may not be of value, even though prospective studies with serial sampling and in SCD-related severe PHT need to be performed in order to further address this issue.

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Circulating endothelial cells: A potential parameter of organ damage in sickle cell anemia?

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ABSTRACT

Objective laboratory tools are needed to monitor developing organ damage in sickle cell disease (SCD). Circulating endothelial cells (CECs) are indicative of vascular injury. We determined whether elevated CEC can be detected in asymptomatic SCD with the Cell Search system and whether the CEC count is related to clinical and blood-based biomarkers of disease severity. Fifteen consecutive clinically asymptomatic HbSS patients and 15 matched HbAA controls were analyzed for CEC counts, laboratory parameters of disease severity (Hb, leukocyte counts, HbF%), plasma levels of markers for endothelial activation (sVCAM-1, vWF:Ag) and of endogenous inhibitors of nitric oxide synthase (asymmetrical dimethylarginine [ADMA]). CEC counts were significantly higher in patients (12 cells/mL, IQR 8–29) as compared to controls (4 cells/mL, 3–10) (p=0.007). CEC counts were significantly higher in patients with pulmonary hypertension (PHT) (p=0.015), and increased with increasing number of affected organs (0–4 involved organs, p=0.002). No significant correlations between CEC and any other laboratory parameter were detected. In conclusion, CECs could prove to be an important new tool for assessing developing vasculopathy and organ damage in SCD.

INTRODUCTION

Sickle cell disease (SCD) is a heterogeneous disorder characterized by chronic hemolysis, increased susceptibility to infections and acute and chronic vaso-occlusive complications culminating in significant morbidity and early death. Both improved supportive care (timely vaccination, penicillin prophylaxis, blood transfusions) and rational drug therapy (hydroxyurea) have contributed to an increasing life expectancy of patients with SCD, with most patients in Western countries surviving into the fourth and fifth decades of life.[1] With this, the impact of accumulating organ damage on patient outcome has become increasingly clear. Both the ongoing vaso-occlusive process with its acute exacerbations, as well as the sequelae of chronic intra-vascular hemolysis, results in continuous endothelial insults, ultimately leading to widespread organ damage. Evidence of endothelial perturbation in SCD comes from animal models and studies in humans demonstrating elevated blood levels of endothelial activation markers, endothelial dysfunction and increased numbers of circulating endothelial cells (CECs).[2-5]

Large patient cohort studies have identified laboratory markers associated with the clinical course of SCD, such as hemoglobin concentrations, leukocyte counts, lactate dehydrogenase (LDH) levels and the fetal hemoglobin percentage (HbF%).[6] Such markers are however, neither sufficiently reliable in predicting developing organ damage in individual patients, nor are they suitable for monitoring the effect of therapeutics in daily clinical practice.[7] Accurate objective laboratory parameters that reflect the rate of developing organ damage in SCD are therefore needed. Attempts to assess the value of monitoring endothelial activation markers in relation to SCD severity have been made.[3] However, most endothelial activation markers are neither specific for endothelial cells (ECs) (e.g., also expressed on or originating from non-ECs), nor specific for endothelial damage or dysfunction.[8] Therefore, measuring CECs may be more accurate for studying *in vivo* endothelial damage as is demonstrated in other disease states characterized by endothelial injury.[8] CECs are elevated and activated in SCD,[5] but their relationship to organ damage in SCD has not been reported.

We performed a pilot study to determine circulating CEC numbers in SCD with a validated automated rare cell analysis system and to assess their relationship to SCD-

related organ damage, laboratory markers of disease severity, markers of endothelial activation and plasma levels of the endogenous nitric oxide synthase (NOS) inhibitor asymmetric dimethylarginine (ADMA).[9]

MATERIALS

Patients

Consecutive patients attending the outpatient hematology clinics of the Erasmus Medical Center (Rotterdam) and the Academic Medical Center (Amsterdam) were screened. Inclusion criteria were high performance liquid chromatography confirmed diagnosis of sickle cell anemia (HbSS) and age ≥18 years. Exclusion criteria were blood transfusions in the preceding three months, any acute SCD-related complication in the preceding 2 weeks, pregnancy, cancer, infection, and connective tissue diseases. Race, age, and sex matched blood donors served as controls (HbAA). Written informed consent was obtained from all subjects. This study was approved by the local Institutional Medical Ethical Review Boards and is in agreement with the Helsinki declaration of 2000.

Clinical data

In the participating centers, sickle cell patients are screened for the presence of organ damage as recently defined and described.[10] From chart review, the following SCD-related complications were scored if assessed within one year of sample collection: *Pulmonary hypertension (PHT)*: tricuspid regurgitation jet flow velocity (TRV) ≥2.5 m/s in rest detected by echocardiography. PHT was considered absent with no or trace TRV. *Micro-albuminuria*: urinary creatinine (mmol/L) to urinary albumin (mg/L) ratio >3.5 (males)/>2.5 (females) confirmed with 24 hour urine collection (microalbuminuria >30 mg/24 h). *Retinopathy*: presence of at least mild non-proliferative retinopathy. *Leg ulcers*: chronic ulcers of the ankle not otherwise explained. The number of admissions for treatment of a *painful crisis* and/or *acute chest syndrome (ACS)* in the year before sample collection was recorded.

Sample collection

Whole blood, ethylenediaminetetraacetic acid (EDTA) blood, citrated blood, and CellSave preserved blood (contains EDTA and a proprietary preservative, Veridex, Raritan, NJ USA) were collected by venipuncture. To avoid contamination with traumatically detached ECs, CellSave tubes were drawn last.

CEC enumeration

The CellTracks AutoPrep and CellSpotter Analyzer II System (both Veridex) were used to count CEC.[9] Four milliliters (mL) of CellSave preserved blood was incubated with ferrofluids coupled to a monoclonal antibody (mAb) against CD146 (clone S-Endo 1), present on ECs, a subset of activated T-cells and melanoma cells. After enrichment, cells were stained with fluorochrome-conjugated mAb: phycoerythrin (PE)-conjugated CD105, present on ECs and certain leukocytes, allophycocyanin (APC)-conjugated CD45, a pan leukocyte marker, and the nuclear dye 4' 6-diamidino-2-phenylindole (DAPI). CECs were immunophenotypically defined as $DAPI^+$, $CD146^+$, $CD105^+$ and $CD45^-$.

Sample analysis

Standard laboratory tests were performed according to local protocols. Serum levels of soluble vascular cell adhesion molecule (sVCAM)-1 were determined using Enzyme-Linked ImmunoSorbent Assay (ELISA) (R&D Systems, Minneapolis, MN). Von Willebrand Antigen (vWF:Ag) and vWF ristocetin cofactor activity (vWF:RCo) were measured in citrated plasma and EDTA plasma concentrations of arginine and ADMA were also determined.[11,12]

Statistical analysis

No published data on CEC distribution as determined with the methods above in SCD were available. Furthermore, no reports on CECs in relation to organ damage in SCD were available and thus the number of CECs that are clinically relevant in relation to organ damage in SCD is presently unknown. Therefore, in this pilot study, a power calculation was not performed. Between group differences were assessed with the Mann–Whitney *U* test. Trends within ordered groups of non-parametric data were

assessed using Cuzick's test for trends.[13] For correlation studies Spearman's rank correlation coefficient (r_s) was determined. Statistical significance was considered with $p<0.05$ (STATA statistical software package 10, StataCorp, College Station, TX, USA).

Results

Results of all measured parameters are shown in Table 1. CEC counts were significantly higher in sickle cell patients. Also, CEC counts were higher in sickle cell patients with PHT as opposed to patients without PHT, with no significant differences observed in relation to other forms of organ damage (Fig. 1). CEC counts increased significantly with increasing numbers of affected organs (Fig. 2). CEC counts were not related to painful crises (data not shown) and no acute chest syndromes or strokes had occurred in the year prior to CEC determination. CEC counts did not differ between patients on hydroxyurea and those who did not use hydroxyurea (data not shown). Additionally, no effect of hydroxyurea was observed on any of the other measured parameters (data not shown) in this cross sectional study. No statistically significant correlations were detected between CEC counts and hemoglobin concentrations, leukocyte counts, LDH concentrations or HbF% (data not shown) or any of the tested markers of endothelial activation (Fig. 3). Additionally, no significant correlations were found between CEC numbers and plasma arginine or ADMA concentrations in either healthy controls (r_s=−0.13, p=0.64) or sickle cell patients (r_s =0.05, p=0.88).

Table 1 | Patient characteristics and laboratory data.

	HbAA (n = 15)		HbSS (n = 15)		*p*-value
Age	24	(21 - 28)	24	(21 - 28)	-
Gender (M:F)	4:14		4:14		-
Hemoglobin (g/L)	14.1	(13.1 - 15.9)	8.7	(7.4 - 10.0)	<0.0001
Leukocytes (x10^9/L)	6.7	(5.8 - 7.3)	8.7	(7.2 - 11.1)	0.003
LDH (IU/L)	144	(139 - 151)	487	(392 - 630)	<0.0001
Creatinin (µmol/L)	75	(55.6 - 95.6)	52	(49 - 57)	<0.0001
HbF (%)	< 1.0%		7.7	(4.4 - 10)	<0.0001
Hydrea use (yes:no)	NA		5:10		
CEC (cells/ml)	4	(3 - 10)	12	(8 - 29)	0.007
vWF:Ag (IU/mL)	0.95	(0.81 - 1.44)	2.01	(1.23 - 2.51)	0.002
vWF:Rco (IU/ml)	0.96	(0.74 - 1.25)	1.54	(1.20 - 1.95)	0.007
VCAM-1 (ng/ml)	921	(765 - 1050)	1879	(1398 - 2568)	0.0001
ADMA (µmol/L)	0.42	(0.39 - 0.45)	0.70	(0.59 - 0.80)	<0.0001
Arginine (µmol/L)	80.8	(72.7 - 90.9)	79.4	(53.0 - 88.7)	NS

Data are shown as medians and corresponding interquartile ranges. NS = not significant.

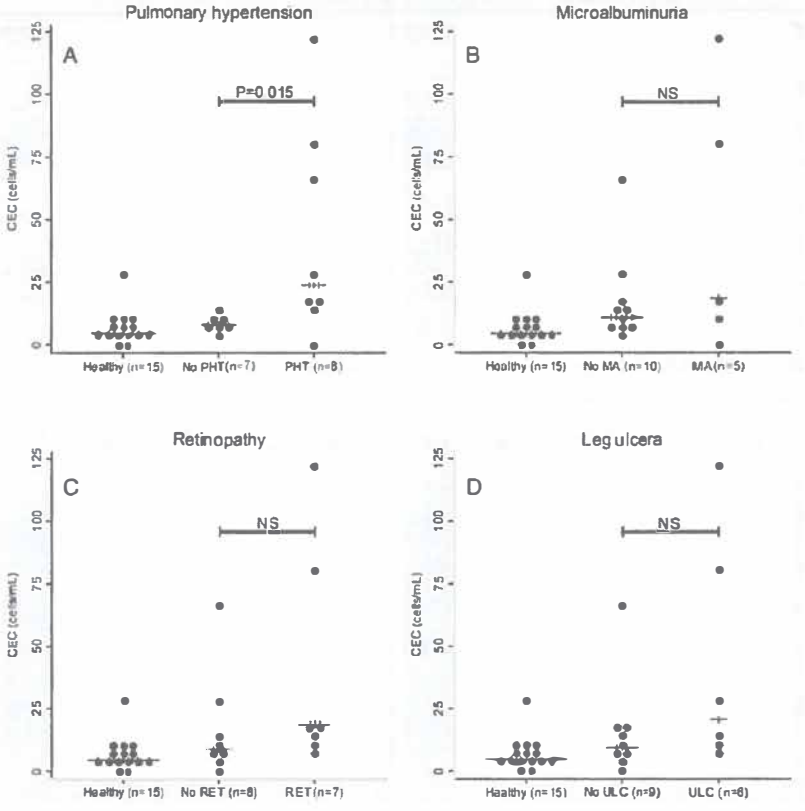

Fig 1. | CEC numbers and relation to specific organ complications. CEC counts were compared using the Mann-Whitney *U* test in SCD patients with and without specific organ complications. Bars indicate median values. NS = not significant.

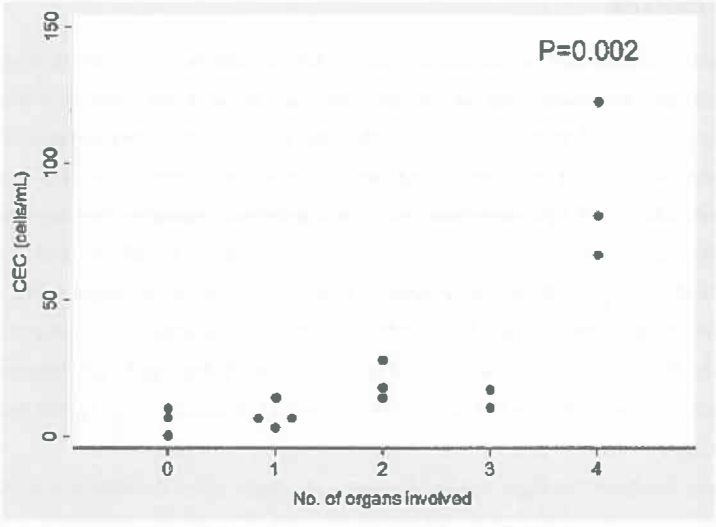

Fig 2. | Relation of CEC and the extent of organ involvement. The significance of trends in CEC counts with increasing organ involvement was assessed using Cuzick's test for trends.

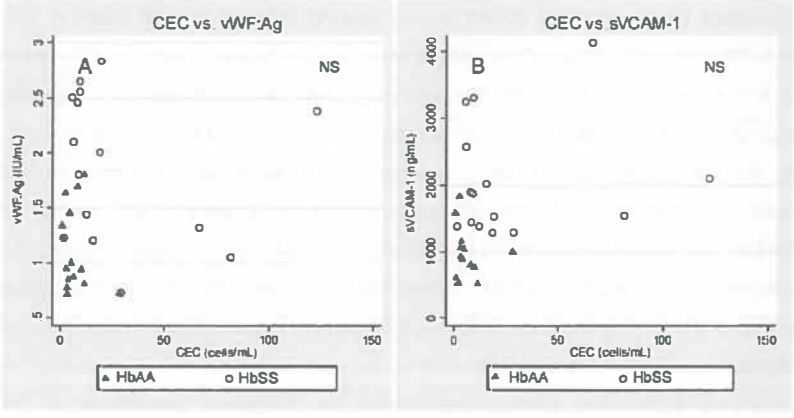

Fig 3. | Correlation of CEC and vWF:Ag and sVCAM-1 levels in patients and controls. Healthy controls: CEC vs. vWF:Ag: r_s = -0.14. p = 0.61. CEC vs. sVCAM-1: r_s = -0.28. p = 0.34. SCD patients: CEC vs vWF:Ag: r_s = -0.27. p = 0.33. CEC vs. sVCAM-1: r_s = -0.01. p = 0.82.

DISCUSSION

Continuous endothelial perturbation culminates in significant organ damage in SCD, and accurate monitoring of this process could be of great value in the study and management of SCD. We detected elevated CEC counts in the same range as previously reported in clinically asymptomatic sickle cell anemia patients using a validated automated CEC detection assay developed to overcome shortcomings of assays based on manual enrichment known to be susceptible to cell loss and to have low reproducibility.[5,14] CEC counts appeared to be highest in those patients with most extensive organ damage. Importantly, patients with minimal organ involvement were characterized by CEC counts comparable to control values. No significant relation was observed between CEC counts and laboratory markers of disease severity, nor to crisis frequency.

Direct EC injury by rigid sickled erythrocytes, high arterial vascular wall shear stress,[8] neutrophil degranulation, present even in the clinically asymptomatic state of SCD,[15] all likely contribute to the detachment of EC from the vessel wall. EC apoptosis can also lead to elevated CECs,[8] however previous work on sickle cell CECs and plasma VEGF levels was suggestive of an anti-apoptotic tone.[16]

Elevated blood levels of sVCAM-1 and vWF:Ag indicate EC activation in SCD, with further increments occurring during acute vaso-occlusive events.[3,17,18] Levels of sVCAM-1 are related to hemolytic rate and either directly or indirectly to SCD-related complications associated with a high hemolytic rate, such as PHT and leg ulcers.[3,19] Also, sVCAM-1 levels have been associated with endothelial dysfunction in SCD as well.[20] Neither sVCAM-1 nor vWF:Ag correlated significantly to CEC counts. EC activation was manifested even in patients with minimal organ damage, whereas CEC counts were comparable to controls in these patients. Importantly, these data suggest that the extent of EC activation/dysfunction is not necessarily related to the extent of EC damage and detachment in SCD.

Recently ADMA has been recognized as an important contributor to the characteristically reduced nitric oxide bioavailability of SCD and its plasma level may be an important biomarker of disease severity, with associations reported to PHT and mortality in adult sickle cell patients.[21,22] Plasma levels are elevated in SCD and,

likely via NOS inhibition, contribute to endothelial activation.[21] However, ADMA levels were not significantly associated to CEC counts in either patients or controls, perhaps suggesting that elevated ADMA levels contribute primarily to endothelial activation but not to endothelial damage.

Several shortcomings should be addressed when interpreting these data. Firstly, our sample size was small, precluding multivariate analysis. Evidently, these findings are preliminary, but the differences in CEC counts between patients without organ damage and those at the other end of the spectrum with all organs affected can be clearly appreciated. Secondly, several clinical complications did not occur in our patients (e.g. acute chest syndromes) and some forms of organ damage were not screened for (e.g. silent ischemic brain injury). Also, we did not monitor patients after blood sampling, so we cannot exclude that acute events were developing at the time of sampling, possibly affecting CEC numbers. Lastly, as we did not characterize the phenotype of CECs, their vascular origin is unknown.

In conclusion, these preliminary data show the feasibility of the employed technique for measuring CEC in SCD. Our findings suggest a possible relation of CEC counts to SCD-related organ damage, and CECs do not seem to be associated with either traditional or novel biomarkers of disease severity. Prospective studies in large patient cohorts addressing chronic organ damage, acute complications and the effect of established therapeutics (e.g. red cell transfusion and hydroxyurea) in relation to longitudinal CEC determinations are warranted to analyze whether CEC can be used as a reliable parameter to monitor developing organ damage in SCD.

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5

Elevated Circulating Stromal-Derived Factor-1 Levels in Sickle Cell Disease

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ABSTRACT

Inflammation and angiogenesis are of importance in the pathophysiology of sickle cell disease (SCD). Recently, the chemokine stromal-derived factor-1 (SDF-1) has been shown to be a key mediator of angiogenesis and inflammation. In this study we determined serum SDF-1 levels in consecutive adult sickle cell patients during the clinically asymptomatic state as well as during painful crises. Serum SDF-1 levels were significantly elevated in HbSS/HbSβ^{o}-thalassemia patients [n = 42; 5,177 pg/ml (2,438–7,246)] compared to HbSC/HbSβ^{+}-thalassemia patients [n = 16; 2,405 pg/ml (1,365–3,047)] and healthy HbAA controls [n = 45; 2,894 pg/ml (2,577–3,334)] (p = 0.001). No significant increments were observed during painful crises (n = 40). SDF-1 levels were significantly higher in SCD patients with pulmonary hypertension (PHT) compared to patients without PHT. Elevated circulating SDF-1 levels occur in patients with SCD and may play a role in the pathophysiology of SCD-related PHT.

INTRODUCTION

Sickle cell disease (SCD) is a severe hemoglobinopathy characterized by an increased susceptibility to infections, chronic hemolytic anemia and continuous microvascular occlusion with acute exacerbations leading to accumulating organ damage and a decreased life expectancy.[1] Both the vaso-occlusive process and intra-vascular hemolysis lead to a complex pathophysiological process characterized by endothelial activation,[2] decreased nitric oxide bioavailability and a recently recognized pro-angiogenic state.[3-7] Elucidation of the complex pathophysiology of SCD is an area of active research in order to identify potential parameters for objectively monitoring disease activity as well as new potential targets for therapy.

The chemokine stromal-derived factor-1 (SDF-1/CXCL12) and its receptor CXCR4 (CD184) have recently been shown to be key mediators of angiogenesis and inflammation.[8,9] Next to direct angiogenic effects, the SDF-1/CXCR4 axis is critically involved in hematopoietic stem cell/endothelial progenitor cell (EPC) recruitment to neo-angiogenic niches supporting the revascularization process of injured or ischemic tissue.[9] In the context of hematopoietic stem cell transplantation, this knowledge has led to promising new and effective stem cell mobilization procedures by using CXCR4 antagonists.

Endothelial cells and pericytes (including smooth muscle cells) express both SDF-1 and CXCR4, which are up-regulated by tissue damage and hypoxia, factors that readily occur in SCD.[10,11] The SDF-1/CXCR4 axis is also involved in endothelial adhesion molecule (such as VCAM-1) induction and in platelet activation,[12,13] which are both important in SCD pathophysiology.[11] Given the above, we set out to examine circulating SDF-1 levels in SCD patients during the clinically asymptomatic state, as well as during a painful crisis. Also, SDF-1 levels were studied in relation to the presence of chronic SCD-related complications.

MATERIALS

Patients

Adult sickle cell patients cared for at the Department of Internal Medicine of the Sint Elisabeth Hospital (Curaçao, Netherlands Antilles), the Slotervaart Hospital (Amsterdam, The Netherlands) and the Department of Hematology of the Academic Medical Center (Amsterdam, The Netherlands) were considered eligible. Blood samples were drawn from consecutively included patients during regular outpatient clinic check-ups as well as during acute painful sickle cell crisis. Blood bank donors served as healthy controls. Written and informed consent was obtained from all patients as well as controls and parents/legal guardians when appropriate. This study was approved by the internal review board of the Academic Medical Center and carried out in accordance with the principles of the Declaration of Helsinki of 1975, as revised in 2000.

SCD-Related Manifestations

In the outpatient clinics adult patients are regularly screened for SCD-related manifestations as previously defined and reported.[14] *Microalbuminuria*: Urinary creatinine (mM) to urinary albumin (mg/l) ratio >3.5 (males) or >2.5 (females) confirmed with 24 h urine collection with micro-albuminuria >30 mg/24 h. *Pulmonary hypertension (PHT)*: Tricuspid regurgitation jet flow velocity 6 2.5 m/s in rest detected by Doppler echocardiography. PHT was considered absent with no or only trace tricuspid regurgitation jet flow velocity. *Retinopathy*: Presence of at least mild non-proliferative retinopathy. *Acute chest syndrome (ACS)*: Defined as previously described occurring between January 2002 and 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 musculoskeletal/abdominal pain not otherwise explained) from January 2002 until January 2007 and categorizing patients into 2 groups (0–1 painful crisis per year and

2 or more painful crises per year). Between-group statistical analysis was confined to comparisons where at least 4 cases were present per group and organ damage had to be screened for at least in the 2 years prior to study inclusion.

Laboratory Parameters

Serum SDF-1 levels were determined according to manufacturer's procedures (R&D Systems, Minneapolis, Minn., USA). All standard laboratory data were obtained during routine outpatient visits at least 4 weeks after the last acute disease-related complication and at least 3 months after last blood transfusions. Fetal hemoglobin percentage was determined by cation exchange high-performance liquid chromatography.

Statistical Analysis

The most severe SCD genotypes (HbSS and HbSβ^o-thalassemia) were grouped together, as were the relatively milder genotypes (HbSC and HbSβ$^+$-thalassemia). Continuous data are presented as medians with their corresponding inter-quartile range. Between multiple group comparisons were analyzed employing the Kruskal-Wallis test. Between 2 group differences were tested with the Mann-Whitney *U* test. For paired sample analysis the Wilcoxon signed rank test was used. For correlation studies the Spearman correlation coefficient (r_s) was calculated. $p<0.05$ were considered statistically significant (SPSS 12.0.2; SPSS ^{Inc}, Chicago, Ill., USA).

RESULTS

Patient and control characteristics as well as serum SDF-1 levels are shown in Table 1. Serum SDF-1 levels were elevated in the HbSS/HbSβ^o-thalassemia patients but did not increase further at presentation with a painful crisis (Table 1). When performing paired sample analysis in patients from whom both steady-state and painful crises samples were available, SDF levels did not differ either between the clinically asymptomatic state or at presentation with a painful crisis (increased in 4, decreased in 7, $p=0.60$). Analysis of serial SDF-1 determinations during evolution of painful crises revealed SDF-1 increments in 4 of 12 painful events (Fig. 1). In both HbSS/HbSβ^o-thalassemia and HbSC/HbSβ$^+$-thalassemia patients, SDF-1 levels were higher in patients with PHT

compared to those without PHT. SDF-1 levels were neither related to other forms of organ damage in our patients nor to hydroxyurea use (data not shown). Also, SDF-1 was not significantly associated with hemoglobin levels, the percentage of fetal hemoglobin, total leukocyte counts or differentials (neither during steady state nor at presentation with a painful crisis) or lactate dehydrogenase levels (data not shown). SDF levels were not related to age in either patients or controls. SDF-1 levels in patients without PHT were also significantly elevated compared to controls (p=0.008).

Discussion

SCD has recently been recognized to be characterized by a pro-angiogenic state,[4-7] likely driven by continuous tissue damage and hypoxia due to both the ongoing vaso-occlusive process as well as the sequelae of chronic intra-vascular haemolysis.[15,16] The SDF-1/CXCR4 axis has been identified as a critical regulator of vascular remodeling and angiogenesis.[9] Our results show circulating SDF-1 levels to be significantly elevated in steady-state HbSS/HbSβ°-thalassemia patients with higher levels in patients affected by PHT, a recently recognized severe SCD-related complication.[17] However, no clear association of circulating SDF-1 levels to acute painful sickle cell crisis could be demonstrated.

Several regulatory pathways could contribute to the elevated SDF-1 levels in HbSS/HbSβ°-thalassemia patients. SDF-1 expression in endothelial cells is induced by transcription factor hypoxia-inducible factor-1α activation which is regulated by the nuclear factor-κB[18,19] and increased nuclear factor- κB activity is typical in the context of the inflammatory state of SCD.[2] Also, considering the reduction of NO bio-availability in SCD,[16] SDF-1 activity during angiogenesis has been shown to be intricately associated with NO bio-availability with,[20] at least in animal models, endothelial NO synthase deficiency associated with increased SDF-1 expression.[21]

In our patients, SDF-1 levels were significantly higher in patients with PHT compared to those without PHT. PHT is nowadays recognized as one of the most important chronic organ complications in adult SCD patients carrying a high risk of early death.[22] Several lines of evidence in animal studies indicate a role of importance of SDF-1 in vascular remodeling in pulmonary arteries.[23,24] Furthermore, an alternate

Table 1 | Patient characteristics and circulating SDF-1 levels

	HbSS/HbSβ°-thalassemia[a]	HbSC/HbSβ⁺-thalassemia[b]	HbAA
Patients	42	16	45
Age	28 (21 - 38)	29 (24 - 38)	45 (36 - 63)
Male/female	25:17	8:8	33:12
Hb, g/dl	9.0 (8.2 - 9.8)	11.2 (10.4 - 12.2)	14.1 (12.3 - 17.4)
SDF-1, pg/ml steady state[c]	5.177 (2.438 - 7.246)	2.405 (1.365 - 3.047)	2.894 (2.577 - 3.334)
SDF-1, pg/ml painful crisis	3.998 (2.605 - 5.623)	2.452 (1.956 - 4.734)	NA

Data are depicted as medians with corresponding interquartile ranges.
[a] HbSS/HbSβ°-thalassemia steady state vs. painful crisis (n = 34): $p = 0.56$; [b] HbSC/HbSβ⁺-thalassemia steady state vs. painful crisis (n = 6): $p = 0.64$; [c] steady state HbSS/HbSβ°-thalassemia vs. HbSC/HbSβ⁺-thalassemia vs. HbAA: $p = 0.001$. NA = not applicable

SDF-1 receptor, CXCR7, has been identified which was recently shown to be up-regulated in hypoxic human pulmonary microvascular endothelial cells accompanied by enhanced SDF-1 expression.[23,25,26] Also, amelioration of hypoxia-induced PHT by statins was associated with down-regulation of circulating SDF-1 levels.[27] Taken together, the association of SDF-1 to SCD-related PHT could suggest a potential role of SDF-1 in PHT pathophysiology.

In light of the regulating mechanisms of SDF-1 expression it was surprising that SDF-1 levels did not increase significantly at presentation with a painful crisis. Both *in vivo* and *in vitro* experiments have shown SDF-1 expression to be strongly correlated to the severity of hypoxia with a relatively short production lag period, and bone marrow ischemia and infarction are central to the sickle cell crisis.[9,28] Even though it remains unexplained at this time why SDF-1 did not increase at presentation with a painful crisis, our observations, including resolution of painful crises with increasing SDF-1 levels (Fig. 1), clearly argue against a role of importance in the uncomplicated painful crisis. SDF-1 levels increased with the development of an ACS in 1 patient and levels returned to baseline with clinical improvement, but none of the other patients with increasing SDF-1 levels developed an ACS. With the observation of SDF-1 increments during morphine-induced central hypoxia during 2 painful crises, these observations may suggest SDF-1 increments to be a biomarker linked to central hypoxia without pathophysiological importance in acute SCD-related complications. However, this needs to be specifically addressed in future studies. In interpretation of these data several factors need to be taken into account. Firstly, healthy controls consisted mainly of relatively older males. However, to the best of our knowledge, SDF-1 levels do not differ between males and females. Although the possibility cannot be eliminated based on our data, we did not detect a relationship of SDF-1 to age in the controls of patients. Secondly, paired sample analysis for steady state and painful crisis SDF-1 levels was limited to a relatively small subgroup. Thirdly, given the small sample size, the findings pertaining to SDF-1 in relation to the presence of organ damage should be considered preliminary, and these need to be reproduced in a larger patient cohort allowing appropriate statistical analysis.

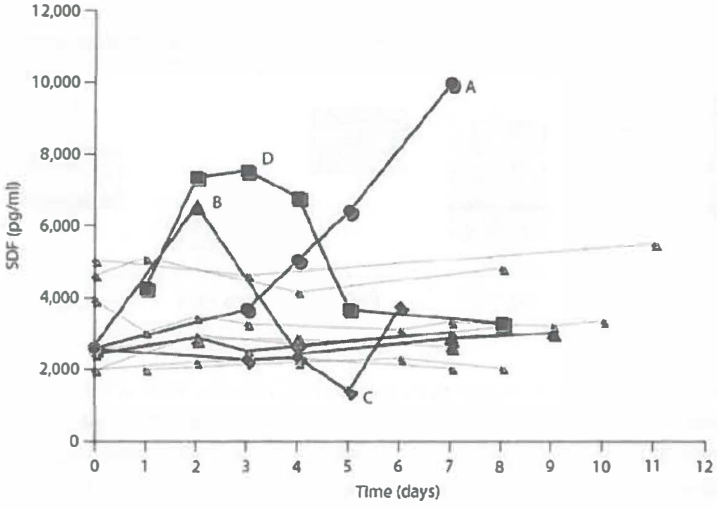

Fig 1. | Serial SDF-1 levels during painful crisis. At least 4 serial blood samples taken during evolution of a painful crisis were available to us during 12 crises occurring in 11 patients. SDF-1 levels remained constant except during crises A (●), B (▲), C (♦) and D (■; remaining crises depicted with small symbol ▲). Crises A and B occurred in the same patient (36-year-old HbSS female) and were uncomplicated painful crises except for several periods oxygen desaturation due to morphine-induced hypoventilation. SDF-1 levels increased whereas the painful crises resolved. Crisis C occurred in a 46-year-old female HbSβ°-thalassemia patient who was admitted with a painful crisis and developed a cholecystitis on day 6 and subsequently underwent urgent laparoscopic cholecystectomy. No post-operative follow-up samples were available for analysis. Crisis D occurred in a 19-year-old HbSS femal. On day 1 shortness of breath developed with an arterial oxygen saturation of 89% and bilateral pulmonary infiltrates. She was treated for an acute chest syndrome with exchange transfusion and started to recover on day 3.

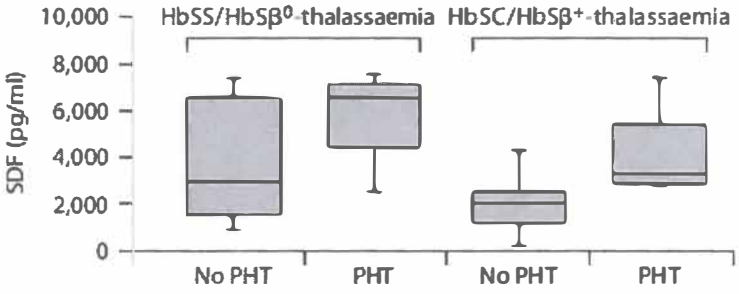

Fig 2. | SDF-1 levels in relation to pulmonary hypertension (PHT) in SCD. Serum SDF-1 levels in patients with and without PHT. SDF-1 levels were significantly higher in HbSS/HbSβ⁰-thalassemia patients with PHT compared to HbSS/HbSβ⁰-thalassemia patients without PHT ($p = 0.02$). In HbSC/HbSβ⁺-thalassemia patients with PHT SDF levels were also higher compared to HbSC/HbSβ⁺-thalassemia patients without PHT ($p = 0.01$).

In conclusion, we demonstrate for the first time elevated levels of SDF-1 in patients with SCD, further supporting the concept of SCD as a pro-angiogenic state. Further research of SDF-1, especially in relation to PHT, is warranted. Also, given emerging data on the role of EPC in SCD pathophysiology,[29] the complex relationship between SDF-1 levels and circulating EPC numbers and function should also now be studied.[30]

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6

Plasma concentrations of asymmetric dimethylarginine, an endogenous nitric oxide synthase inhibitor, are elevated in sickle cell patients but do not increase further during painful crisis

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ABSTRACT

Plasma concentrations of asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase inhibitor, are elevated in the clinically asymptomatic state of sickle cell disease (SCD). However, the role of ADMA during vaso-occlusive complications has not been defined. ADMA concentrations were determined in HbSS (n=43) and HbSC (n=25) patients with healthy blood donors (HbAA) as controls. In the clinically asymptomatic state ADMA concentrations were elevated in sickle cell patients as compared to healthy controls (HbSS 0.70 µmol/L, HbSC 0.54 µmol/L, HbAA 0.39 µmol/L) (p<0.001). Yet plasma ADMA concentrations did not increase further at presentation with a painful crisis implicating no role of primary importance during vaso-occlusive crises.

INTRODUCTION

Vaso-occlusion leads to accumulating ischemic organ damage and a decreased life expectancy in patients with sickle cell disease (SCD).[1] Over the last years the importance of a reduced nitric oxide (NO) bioavailability in the complex pathophysiology of several SCD-related complications such as pulmonary hypertension (PHT) and stroke has become increasingly clear.[2,3] NO plays a central role in vascular homeostasis; it is a potent vasodilator which modulates endothelial activation, inhibits smooth muscle cell proliferation and migration, limits platelet aggregation, and ischemia-reperfusion injury.[2] During the clinically asymptomatic state NO bioavailability is reduced in sickle cell patients due to the scavenging of NO by cell-free hemoglobin released from hemolyzed sickle erythrocytes,[4] as well as due to increased arginase activity.[5] During acute vaso-occlusive events such as painful crises and acute chest syndromes a further decrease of NO bioavailability has been demonstrated.[6,7]

We recently reported elevated plasma asymmetric dimethylarginine (ADMA) concentrations during the clinically asymptomatic state in SCD.[8] ADMA, as well as N^G,N'^G- dimethyl-L-arginine (symmetric dimethylarginine or SDMA) and N^G-monomethyl-L-arginine (L-NMMA), derive from irreversible post-translational methylation of guanidino nitrogens of arginine residues and are released as free amino acids upon proteolysis. ADMA competitively inhibits NO synthase (NOS), thereby reducing the conversion of arginine to citrulline thus limiting NO production. SDMA is not a NOS inhibitor, and the plasma concentration of ADMA is 10 times greater than that of L-NMMA. ADMA is degraded by dimethylarginine dimethylaminohydrolase (DDAH) which hydrolyzes ADMA to dimethylamine and citrulline. Hypoxia and elevated levels of pro-inflammatory cytokines may inhibit or down-regulate DDAH.[9-12] As both hypoxia and inflammation readily occur during vaso-occlusive events in SCD, further increments in ADMA concentrations can be expected. We therefore set out to investigate whether ADMA concentrations increase during acute vaso-occlusive complications in SCD.

MATERIALS AND METHODS

Patients and controls

Sixty-eight EDTA anticoagulated plasma samples were available from 52 sickle cell patients during the clinically asymptomatic state and 91 samples were available from 35 sickle cell patients during a painful crisis (35 samples taken at presentation with a painful crisis and 56 follow-up samples during evolution of a painful crisis). Blood samples were prospectively collected over a 7-year period at both the outpatient clinic and the emergency ward of the Sint Elisabeth Hospital in Curaçao, the Netherlands Antilles. The clinically asymptomatic state was defined as being free of pain at least 4 weeks after the last acute SCD-related complication. A painful crisis was defined as an episode of acute pain in the extremities and/or abdomen not otherwise explained.[19] None of the patients were on any kind of treatment (apart from folic acid supplementation), nor had they received blood transfusions during the 3 months prior to sample collection. Thirty-five race (but not age or sex) matched, blood donors served as healthy controls. Patients and controls gave written informed consent before sample collection. This study was approved by the local Ethical Review Board and is in agreement with the Helsinki Declaration of 2000.

Samples and analysis

Standard blood counts and clinical biochemistry (e.g. creatinine and lactate dehydrogenase (LDH) levels) were determined according to local protocols. EDTA anticoagulated blood samples were immediately centrifuged at 1700g for 10 min. Plasma was collected and aliquots were stored at -70°C until further analysis. Plasma concentrations of arginine, ADMA, and SDMA were measured employing high-performance liquid chromatography with fluorescence detection as previously described, with modified chromatographic separation conditions.[20,21] The inter-assay and intra-assay coefficients of variation were <3% and <1.5%, respectively, for all compounds. The upper limits (*P*97.5) of the reference range are 0.63 and 0.73 µmol/L for ADMA and SDMA, respectively.[22]

Data analysis and statistics

All data are presented as medians with corresponding interquartile ranges. For between multiple group comparisons the Kruskal-Wallis test was employed. The Mann-Whitney *U*-test was used for comparison between two groups. For paired sample analysis the Wilcoxons' signed rank test was used. Statistical software Package for the Social Science (SPSS), version 14.0, SPSS ^{Inc}, Chicago, Ill (USA) was used and $p<0.05$ was considered statistically significant.

RESULTS

Plasma ADMA and SDMA concentrations were significantly elevated during the clinically asymptomatic state in both HbSS and HbSC patients as compared to healthy controls (Table 1). At presentation with a painful crisis, plasma ADMA concentrations did not increase further in HbSS or HbSC patients, but in HbSS patients the arginine/ADMA ratios significantly decreased due to significant reductions in arginine concentrations (Table 2). Samples were available of 14 patients in both the clinically asymptomatic state and at presentation with a painful crisis. No statistically significant change in ADMA, arginine or arginine/ADMA could be detected in paired sample analysis (data not shown). Also, no consistent pattern could be detected of ADMA and arginine in four patients of whom at least four serial samples were available during the evolution of a painful crisis until crisis abatement (data not shown).

Table 1 | Steady state patient and control demographics and ADMA, SDMA and Arginine concentrations

	HbSS (n = 33)	HbSC (n = 19)	HbAA (n = 35)	*p*-value
Age (years)	33 (9 - 60)	32 (17 - 45)	47 (27 - 61)	<0.001
Male:female	18:15	8:11	33:2	<0.001
Hemoglobin (g%)	8.3 (6.9 - 8.8)	10.1 (8.6 - 11.8)	15.3 (13.9 -16.4)	<0.001
Creatinin mg/L	8.8 (6.9 - 13.6)	8.0 (7.1 - 9.4)	11.3 (9.9 - 12.0	<0.001
LDH (U/L)	588 (350 - 944)	320 (282 -393)	134 (125 - 156)	<0.001
ADMA (µmol/L)	0.70 (0.60 - 0.83)	0.54 (0.47 - 0.58)	0.39 (0.36 - 0.45)	<0.001
Arginine (µmol/L)	58.5 (39.5 - 79.0)	70.8 (59.8 - 80.4)	72.9 (55.9 - 89.3)	0.04
Arginine/ADMA ratio	80.5 (55.4 -109.2)	147.2 (107.9 -168.9)	183.7 (140.5 - 222.6)	<0.001
SDMA (µmol/L)	0.55 (0.45 - 0.76)	0.49 (0.43 - 0.55)	0.42 (0.36 - 0.46)	<0.001

Patient characteristics data are depicted as median with interquartile ranges. The Kruskal-Wallis test was employed for between group analysis.

Table 2 | ADMA, SDMA and Arginine concentrations in the clinically asymptomatic state and at presentation with a painful crisis

	HbSS			HbSC		
	Asymptomatic state (n = 33)	Painful crisis (n = 24)	*p*-value	Asymptomatic state (n = 19)	Painful crisis (n = 11)	*p*-value
ADMA (µmol/L)	0.70 (0.63 -0.83)	0.66 (0.59 - 0.77)	0.5	0.54 (0.47 - 0.58)	0.55 (0.49 -0.61)	0.91
Arginine (µmol/L)	58.5 (39.5 -79)	52.3 (37.5 -70.8)	0.01	70.8 (59.8 - 80.4)	58.4 (37.1 -91.4)	0.4
Arginine/ ADMA ratio	80.5 (55.4 - 109.2)	71.0 (53.4 - 108.2)	0.03	147.2 0 (107.9 - 168.9)	104.3 (68.0 - 162.8)	0.29
SDMA (µmol/L)	0.55 (0.45 -0.76)	0.51 (0.43 - 0.65)	0.6	0.49 (0.43 - 0.55)	0.51 (0.43 - 0.61)	0.58

Data are depicted as median with interquartile ranges. The Mann-Whitney *U* test was employed for between group analysis.

DISCUSSION

Elevated plasma ADMA concentrations lead to a reduced NO bioavailability through NOS inhibition and ADMA is known to be involved in several vascular disease states such as atherosclerosis, and preeclampsia.[11] In this study, we determined whether ADMA concentrations increase during painful crises in SCD.

As expected, plasma ADMA concentrations were higher in sickle cell patients as compared to healthy controls, with highest values detected in HbSS patients. No changes were observed in the absolute ADMA concentration (or SDMA concentration) at presentation with a painful crisis, but a significant decrease was seen in the arginine/ADMA ratios in HbSS patients at presentation with a painful crisis due to a decrease in the plasma arginine concentration (likely as result of increased NOS and/or arginase activity).[2] No clear pattern of ADMA or arginine could be detected with either paired sample analysis or with analysis of serial measurements. Therefore, a primary role of ADMA in the pathophysiology of the painful sickle cell crisis seems unlikely based on these findings.

The main pathway of ADMA metabolism is degradation by DDAH,[12] with a small fraction of ADMA excreted by the kidneys. As DDAH is inhibited during an inflammatory response,[9] we would have expected plasma ADMA increments during painful crises. Why this does not seem to occur is not clear at this time. All sickle cell patients immediately receive oxygen, intravenous morphine, and saline at admission to the emergency ward prior to blood sample collection. Usually samples are collected within an hour upon arrival at the emergency ward. Hypoxia leads to DDAH down-regulation,[10] but during an uncomplicated painful crisis, hypoxia is unlikely to occur to a significant extent in the lungs, kidney or liver (organs characterized by DDAH activity), perhaps in part explaining the lack of ADMA increments during sickle cell crisis. Furthermore, it is unknown whether oxygen supplementation increases DDAH activity. Even if this would be the case, it is unlikely that this would lead to ADMA reduction on such short notice. It is also unlikely that saline administered to our patients before sample collection (250 cc at most) has led to ADMA dilution, thereby masking an ADMA increment. As arginine inhibits DDAH activity,[13] the documented arginine reduction could in part explain the lack of ADMA increments.

It is unlikely that reduced renal function is the sole explanation for the increased ADMA concentration in SCD during the clinically asymptomatic state, as SDMA, which is mainly cleared by renal excretion,[12] was not elevated to the same extent as ADMA. Furthermore, even though serum creatinine levels overestimate renal function in SCD,[14] creatinine concentrations were higher in healthy controls as compared to sickle cell patients. Plausible causes of elevated ADMA concentrations in SCD may be decreased DDAH activity and increased release of free ADMA (and SDMA) by proteolysis associated with the increased erythrocyte turnover (ADMA concentrations are reported highest in sickle cell patients with lowest hemoglobin concentrations).[8] Furthermore, SCD is characterized by chronically elevated vascular wall shear stress[15] which is known to induce expression of endothelial type-I protein arginine methyltransferase, a catalyst of arginine methylation.[16]

In interpreting these data several limitations should be considered. Firstly, paired sample analysis was possible in a limited number of patients and the sample size of our study was relatively small. Secondly, ADMA concentrations are increased in primary PHT and PHT occurs in approximately 30% of adult sickle cell patients.[17,18] At the time these samples were drawn it was unknown if patients had PHT. Even though it is likely that several patients would have had PHT, the role of ADMA in SCD-related PHT is not known. Therefore, a potential confounding effect on the main results of this study is currently unclear. Thirdly, most healthy controls were male and older than the patient group. Even though plasma ADMA concentrations are known to be higher in post menopausal women than in men and increase with age, this does not influence our main findings.

In conclusion, plasma ADMA concentrations are elevated in patients with SCD but do not seem to increase further at presentation with a painful crisis, arguing against a role of primary importance of ADMA in the painful sickle cell crisis.

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Association of asymmetric dimethylarginine with sickle cell disease-related pulmonary hypertension

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Introduction

Pulmonary hypertension (PHT) occurs in approximately 30% of adult sickle cell patients and is associated with a high risk of early death. Hemolysis driven reductions in nitric oxide (NO) bioavailability resulting from NO scavenging by cell free hemoglobin and increased arginase activity are of importance in the pathophysiology of SCD-related PHT.[1]

Elevated plasma concentrations of asymmetric dimethylarginine (ADMA) contribute to limiting NO bioavailability in SCD.[2] ADMA and symmetric dimethylarginine (SDMA) derive from the irreversible post-translational methylation of arginine residues by protein arginine methyltransferases (PRMT) and are released as free amino acids upon proteolysis. ADMA (but not SDMA) competitively inhibits NO synthase (NOS) enzymes, thereby limiting NO production. ADMA is degraded by dimethylarginine dimethylaminohydrolases (DDAH) whereas SDMA is mainly cleared renally.[3] Elevated plasma ADMA concentrations occur in several forms of PHT and are associated to PHT outcome.[4,5] We investigated whether ADMA concentrations are associated with PHT in SCD.

Materials and Methods

Serum and EDTA plasma samples were available from adult sickle cell patients consecutively screened for PHT with echocardiography as previously reported.[6] Mild and moderate-severe PHT are defined as tricuspid regurgitant jet flow velocity (TRV) of 2.5-2.9 m/s and TRV≥3 m/s respectively, with pulmonary-artery pressures considered normal in patients with trace or no tricuspid regurgitation (with TRV assigned 1.3 m/s).[1] Plasma concentrations of ADMA, SDMA, amino acids and serum soluble vascular cell adhesion molecule (sVCAM)-1 levels were determined as previously described.[2,7] For analysis, HbSS and HbSβ^0-thalassemia patients were grouped together, as were HbSβ$^+$-thalassemia and HbSC patients. *p*-values <0.05 were considered statistically significant (SPSS 12.0.2, SPSS ^{Inc}, Chicago, IL, USA). The study was carried out in accordance with the principles of the Declaration of Helsinki.

Two out of 19 PHT patients had moderate-severe PHT. Hydroxyurea use did not differ between patients with and without PHT and no patients used anticoagulation,

calcium antagonists, endothelin receptor blockers or sildenafil. Between-group comparisons were only performed in HbSS/HbSβ^o-thalassemia patients as only 3 HbSC/HbSβ$^+$-thalassemia patients had PHT of whom one had blood samples drawn.

RESULTS AND DISCUSSION

ADMA concentrations in patients without PHT were high compared to previously reported values in healthy race-matched controls. Irrespective of PHT, HbSS/HbSβ^o-thalassemia patients were characterized by lower hemoglobin, higher LDH, ADMA and sVCAM-1 concentrations than HbSC/HbSβ$^+$-thalassemia patients (all p<0.001). ADMA and sVCAM-1 were higher in HbSS/HbSβ^o-thalassemia patients with PHT than those without PHT, with a significant correlation between ADMA and TRV as well (Table 2). sVCAM-1 and hemoglobin were significantly correlated to TRV in HbSS/HbSβ^o-thalassemia patients (r_s=0.49, p=0.002, r_s =-0.30, p=0.04, respectively). SDMA, but not ADMA, was significantly correlated to GFR (r_s =-0.66, p<0.001, r_s =-0.08, p=0.60, respectively) in HbSS/HbSβ^o-thalassemia patients. Given the relation between hemolysis and methylarginine concentrations, it is likely that the hemolytic rate is an important determinant of their production in SCD (likely due to the increased protein turn-over in the stress erythropoiesis), also explaining the higher concentrations in HbSS/HbSβ^o-thalassemia patients. A relative decrease in renal function (generally more evident in HbSS/HbSβ^o-thalassemia patients) could contribute especially to SDMA elevations. Contributing factors related to the pulmonary vasculature could be shear stress induced PRMT activity[8] and hypoxia induced DDAH downregulation.[9] Although difference in ADMA between patients with and without PHT seems modest, even small increases in extra-cellular ADMA lead to significant intra-cellular NOS inhibition through preferred cellular ADMA uptake over arginine.[3] Indeed, plasma ADMA concentrations ≥0.64 µmol/L are associated with strongly reduced pulmonary artery endothelial NOS expression and early death in PHT patients.[5] Based upon the strong correlation of sVCAM-1 to ADMA, it would be interesting to hypothesize that chronic hemolysis induced ADMA elevations significantly contribute to endothelial activation and dysfunction in SCD via NOS inhibition, and that patients with higher ADMA concentrations are more prone to develop a vasculopathy leading to complications such as PHT over time.

Arginase activity (reflected by arginine to ornithine ratios) is elevated in sickle cell patients with moderate-severe PHT but, in agreement with previous studies,[1,10] did not differ between patients with mostly mild PHT and those without PHT. Conceding the fact that we did not determine plasma arginase activity directly, these data suggest that ADMA could play a role of pathophysiological importance at a relatively earlier stage than arginase activity.

The relatively small number of patients needs to be taken into account when interpreting these data and no conclusions can be drawn about HbSC/HbSβ$^+$-thalassemia patients. Also, right heart catheterization remains the gold standard diagnostic test for PHT and is recommended in sickle cell patients with moderate-severe PHT detected with echocardiography. However, given the excellent correlation between pulmonary artery pressure and TRV in SCD,[1] and the fact that an elevated TRV is the result of solely left-sided heart disease in only a minority of cases,[11] the lack of right heart catheterization is unlikely to have significantly affected our results. Lastly, our data are largely limited to patients with mild PHT. Nonetheless, mortality is high in these patients and plasma ADMA concentrations were well in the range associated with death in other forms of PHT.[4,5]

Taken together, our data identify an association of plasma ADMA concentrations to PHT in SCD, possibly identifying a novel factor of importance in its pathophysiology. Also, ADMA induced limitation of NO production may well provide an important new mechanistic link between hemolysis and the characteristic endothelial activation of SCD.

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Table 1 | Demographics and laboratory parameters in sickle cell patients with and without pulmonary hypertension

	HbSS (n=40) / HbSβ^0-thalassemia (n=6)		
	PHT -	PHT +	P
N	28	18	
Age (years)	33 (21-44)	28 (22-52)	0.80
Male:female	6:22	5:13	
TRV (m/s)	2.0 (1.3-2.3)	2.7 (2.6-2.8)	
PAP (mmHg)	21 (12-28)	34 (32-43)	
Hb (mmol/L)	5.7 (5.0-6.2)	4.9 (4.2-5.9)	0.05
HbF (%)	10.6 (6.1-18.3)	5.9 (2.2-14.1)	0.13
LDH (U/L)	369 (300-515)	575 (388-846)	0.02
GFR (mL/min)	151 (120-195)	120 (66-172)	0.10
ADMA (µmol/L)	0.57 (0.52-0.65)	0.63 (0.58-0.79)	0.01
SDMA (µmol/L)	0.47 (0.42-0.55)	0.51 (0.47-0.83)	0.07
Arginine (µmol/L)	45 (32-56)	46 (41-62)	0.26
Ornithine (µmol/L)	56 (42-66)	56 (45-75)	0.41
Citrulline (µmol/L)	23 (16-32)	27 (20-32)	0.66
Proline (µmol/L)	208 (162-257)	209 (176-234)	0.84
Arginine/ornithine	0.84 (0.66-1.0)	0.93 (0.72-1.15)	0.45
Arginine/citrulline	1.87 (1.66-2.49)	1.98 (1.43-2.65)	0.84
Arginine/proline	0.23 (0.18-0.31)	0.25 (0.19-0.35)	0.41
sVCAM-1 (ng/mL)	1089 (801-1239)	1542 (1119-1880)	0.007

Data are presented as medians with their corresponding interquartile ranges. A *p*-value <0.05 is considerd statistically significant. *Right ventricular systolic pressure was estimated based on the modified Bernoulli equation (1) and considerd to be equal to the systolic pulmonary artery pressure (sPAP) in absence of right ventricular outflow obstruction. ** Glomerular Filtration Rate (GFR) calculated with Cockcroft and Gault-formula (males: creatinine clearance = 1.23xweight x (140-age/serum creatinine, females: creatinine clearance = 1.03xweight x (140-age)/serum creatinine).

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Plasma asymmetric dimethylarginine concentrations in sickle cell disease are related to the hemolytic phenotype

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ABSTRACT

Asymmetric dimethylarginine (ADMA) is associated with pulmonary hypertension (PHT) in sickle cell disease (SCD). We studied the relationship of ADMA to other SCD-related complications. Plasma ADMA and associated parameters were determined in 52 HbSS/HbSβ^{o}-thalassemia and 24 HbSC/HbSβ^{+}-thalassemia patients. As expected ADMA levels were higher in HbSS/HbSβ^{o}-thalassemia patients with PHT (p=0.018), but also in those with other hemolysis-associated complications such as leg ulcers (p=0.012), cholelithiasis (p=0.008) and priapism (p=0.02) compared with counterparts without these complications. ADMA levels did not differ between patients with and without other disease-related complications such as retinopathy and avascular osteonecrosis. Higher ADMA concentrations therefore seem to be associated to the hemolytic phenotype of SCD.

INTRODUCTION

In sickle cell disease (SCD) reduced nitric oxide (NO) bioavailability leads to endothelial dysfunction and is associated with several vascular disease states, such as pulmonary hypertension (PHT), renal dysfunction, priapism, leg ulcers and stroke.[1,2] Chronic hemolysis is considered the driving force behind the NO shortage characteristic of SCD.[2,3] Circulating free hemoglobin released from red cells scavenges NO,[4] while increased arginase activity secondary to chronic hemolysis also contributes by limiting arginine availability for NO synthesis.[5] Hence, the more severe the hemolytic anemia, the greater the NO shortage. As such, two extremes within the SCD spectrum can be appreciated, i.e. patients with severe hemolysis characterized by a high incidence of PHT, priapism, leg ulcers and stroke (hemolytic phenotype), and those with low rates of hemolysis and high incidences of painful crises and acute chest syndrome (ACS) (vaso-occlusive phenotype).[6]

We previously reported that plasma asymmetric dimethylarginine (ADMA) concentrations are elevated in SCD.[7] ADMA is an endogenous NO synthase (NOS) inhibitor that contributes to the chronic NO shortage in SCD. The ADMA levels are associated with the hemolytic rate, endothelial activation and with SCD-related PHT.[7,8] Moreover, plasma ADMA concentrations were recently identified as a risk factor for early death in SCD.[9] However, whether ADMA is also related to other hemolysis-related SCD complications is currently unknown. Therefore, we studied whether plasma ADMA concentrations are associated with hemolysis associated complications of SCD other than PHT in a well defined patient population.

MATERIALS AND METHODS

Patients

This study was conducted between 2005 and 2007. Adult sickle cell patients cared for at the department of Internal Medicine of the Sint Elisabeth Hospital (Curaçao, Netherlands Antilles), the Slotervaart Hospital (Amsterdam, the Netherlands) and the department of Hematology of the Academic Medical Centre (Amsterdam, the Netherlands), were considered eligible. Blood samples were drawn from consecutively included patients during regular outpatient visits. Written and informed consent were

obtained from all patients. This study was approved by the internal review board of the Academic Medical Centre and carried out in accordance with the principles of the Declaration of Helsinki of 1975, as revised in 2000.

SCD-related manifestations

In the outpatient clinics adult patients are regularly screened for SCD-related manifestations as previously defined and reported.[10] The following data were taken from the medical records: *microalbuminuria*: urinary albumin (mg/L) to urinary creatinine (mmol/L) ratio in mg/mmol >3.5 (males)/>2.5 (females) confirmed with the 24 hour urine collection with micro-albuminuria >30 mg/24 h. *PHT*: tricuspid regurgitation jet flow velocity (TRV) ≥2.5 m/s in rest detected by Doppler echocardiography. PHT was considered absent when no or only trace TRV is detected. *Retinopathy*: presence of at least mild non-proliferative retinopathy. *Cholelithiasis*: presence of gallstones (ultrasound) or previous cholecystectomy because of cholecystolithiasis. *ACS*: defined as previously described occurring between January 2002 and January 2007.[11] *Symptomatic avascular osteonecrosis (AVN)*: local pain and reduced function with 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. *Painful crises*: the cumulative number of admissions for painful crises (defined as typical musculo-skeletal/abdominal pain not otherwise explained) from January 2002 until January 2007 was used to categorize patients into 2 groups: 0–1 painful crisis per year and 2 or more painful crises per year.

Laboratory determinations

EDTA anti-coagulated plasma of 76 adult sickle cell patients was available to us.[10] These were drawn between 2005 and 2007 and stored at −80 °C and never thawed before. Plasma concentrations of arginine, ADMA and symmetric dimethylarginine (SDMA)

were measured by high-performance liquid chromatography with fluorescence detection as previously described,[12] with modified chromatographic separation conditions.[13] The inter-assay and intra-assay coefficients of variation were <3% and <1.5% respectively for arginine, ADMA and SDMA. The upper limits (*P*97.5) of the reference range are 0.63 and 0.73 µmol/L for ADMA and SDMA, respectively.[14] Quantitative brain natriuretic peptide (BNP) and N-terminal brain natriuretic peptide (NTproBNP) levels were measured in EDTA plasma with a microparticle enzyme immunoassay (Abbott Diagnostics) and an electrochemiluminescence immunoassay (Roche Diagnostics), respectively. Serum soluble vascular cell adhesion molecule (sVCAM)-1 levels were determined according to the manufacturer's procedures (R&D Systems USA). Standard blood counts and clinical biochemistry (e.g. hemoglobin (Hb), leukocytes, creatinine and lactate dehydrogenase levels (LDH)) were determined according to local protocols. Glomerular filtration rate (GFR) was calculated with the Cockroft and Gault formula.

Statistical analysis

The most severe SCD genotypes (HbSS and HbSβ^o-thalassemia) were grouped together, as were the relatively milder genotypes (HbSC and HbSβ$^+$-thalassemia). Continuous data are presented as medians with their corresponding interquartile range (IQR). Between-group statistical analysis were confined to comparisons where at least four cases were present per group, and organ damage had to be screened for in the two years prior to study inclusion. Between 2 groups differences were tested with the Mann–Whitney *U* test. For correlation studies the Spearman correlation coefficient (r_s) was calculated. *p*-values <0.05 were considered statistically significant (SPSS 12.0.2, SPSS ^{Inc}, Chicago, IL).

RESULTS

Patient characteristics and clinical data are shown in Table 1. Apart from retinopathy, cholelithiasis and α-thalassemia the frequency of other complications was too low in the HbSC/HbSβ$^+$-thalassemia group to allow statistical analysis. There were no statistically significant differences in ADMA concentrations in the analyzed complications of the HbSC/HbSβ$^+$-thalassemia group (Table 2). In the HbSS/HbSβ^o-thalassemia group the frequency of stroke was too low to allow statistical analysis.

Plasma ADMA concentrations were significantly higher in patients with PHT and in patients with a history of cholelithiasis, leg ulcers and priapism. SDMA concentrations were significantly higher in patients with micro-albuminuria and PHT and lower in patients with α-thalassemia. Arginine and the arginine/ADMA ratio were significantly lower in patients with a history of ACS (Table 2). ADMA and SDMA correlated inversely to Hb (r_s=−0.47, p=0.001 and r_s=−0.51, p=0.001 respectively) and positively to LDH (r_s=0.54, p<0.001 and r_s=0.31, p=0.03 respectively). SDMA, but not ADMA, was inversely correlated to the GFR (r_s=−0.66, p<0.001, r_s=−0.08, p=0.60, respectively). ADMA was positively correlated to BNP (r_s=0.351, p=0.011). SDMA and sVCAM-1 were both positively correlated to BNP (r_s=0.458, p=0.001 and r_s=0.573, p<0.001 respectively) and NTproBNP (r_s=0.475, p<0.001 and, r_s=0.538, p<0.001 respectively). BNP was correlated to Hb (r_s=−0.617, p<0.001), LDH (r_s=0.64, p<0.001) and GFR (r_s=−0.318, p=0.028). NTproBNP was correlated to Hb (r_s=−0.411, p=0.002), LDH (r_s=0.380, p=0.006) and GFR (r_s=−0.390, p=0.006).

Table 1 | Legenda
Results are presented as medians with their corresponding IQR.
ns = not significant
GFR = Glomerular filtration rate; LDH = lactate dehydrogenase; sVCAM-1 = soluble vascular adhesion molecule-1; BNP = brain natriuretic peptide; NTproBNP = N-terminal pro-b-type natriuretic peptide; ADMA = asymmetric dimethylarginine; SDMA = symmetric dimethylarginine.
[a] Fraction in percentages.
[b] Cockcroft and Gault-formula (males: creatinine clearance = 1.23xweight x (140-age/serum creatinine, females: creatinine clearance = 1.03xweight x (140-age)/serum creatinine).

Table 1 | Demographics and laboratory data of sickle cell patients

	HbSS/HbSβ°-thalassemia (n = 52)	HbSC/HbSβ⁺-thalassemia (n = 24)	*p*
Age (years)	28 (21 -47)	30 (23-38)	ns
Male:female	14:38	13:11	
Microalbuminuria[a]	17/42 (40.5)	1/22 (4.5)	
PHT[a]	19/50 (38.0)	2/23 (8.7)	
Retinopathy[a]	12/44 (27.3)	14/24 (58.3)	
Cholelithiasis[a]	34/48 (70.8)	4/20 (20.0)	
ACS[a]	21/49 (42.9)	1/24 (4.2)	
AVN[a]	6/48 (12.5)	1/24 (4.2)	
Leg ulcers[a]	5/48 (10.4)	0/23 (0.0)	
Priapism[a]	5/14 (35.7)	0/11 (0.0)	
Stroke[a]	2/52 (3.8)	0/24 (0.0)	
Crises ≥ 2/year[a]	7/49 (14.3)	2/24 (8.3)	
α-thalassemia[a]	15/36 (41.7)	7/18 (38.9)	
GFR (ml/min)[b]	151 (113 - 182)	125 (114 - 139)	ns
LDH (U/L)	439 (330 -858)	232 (202 - 291)	<0.001
sVCAM-1 (ng/ml)	1122 (964 - 1357)	746 (622 - 971)	<0.001
BNP (pg/ml)	49 (26 - 106)	17 (22 - 44)	<0.001
NTproBNP (pg/ml)	101 (53 -158)	39 (22 - 44)	<0.001
ADMA (μmol/L)	0.60 (0.54 - 0.68)	0.49 (0.46 - 0.57)	<0.001
SDMA (μmol/L)	0.49 (0.42 - 0.58)	0.46 (0.44 - 0.56)	ns
Arginine (μmol/L)	56 (47-71)	68 (56 -79)	ns
Arginine/ADMA ratio	91 (72 - 115)	132 (113 - 165)	0.001

Table 2 | Parameters in the HbSS/HbSβ°-thalassemia patients with and without SCD-related complications

	without	with	p
Microalbuminuria			
ADMA	0.59 (0.54-0.65)	0.58 (0.53-0.71)	ns
Arginine	53.6 (46.6-70.9)	55 (46.2- 65.6)	ns
SDMA	0.46 (0.42-0.50)	0.56 (0.47-0.82)	0.021
sVCAM-1	1072 (893.6-1257.2)	1165.6 (889.1-1342.6)	ns
PHT			
ADMA	0.57 (0.53-0.63)	0.61 (0.58-0.75)	0.018
Arginine	51.4 (43.4-63.7)	61.0 (48.6-71.1)	ns
SDMA	0.47 (0.41-0.53)	0.51 (0.46-0.83)	0.039
sVCAM-1	1083.6 (807.7-1200.7)	1440.1 (1072.0-1877.8)	0.023
Retinopathy			
ADMA	0.60 (0.56-0.68)	0.55 (0.51-0.73)	ns
Arginine	55.9 (46.8-69.8)	56.1 (49.4-68.9)	ns
SDMA	0.49 (0.44-0.57)	0.56 (0.44-0.74)	ns
sVCAM-1	1144.5 (893.6-1451.5)	1114.6 (1072.0-1328.6)	ns
Cholelithiasis			
ADMA	0.56 (0.53-0.59)	0.63 (0.58-0.71)	0.008
Arginine	55.3 (46.8-69.8)	54.2 (45.3-56.4)	ns
SDMA	0.48 (0.43-0.60)	0.49 (0.41-0.58)	ns
sVCAM-1	1049.0 (798.4-1285.0)	1164.2 (985.8-1546.4)	ns
ACS			
ADMA	0.60 (0.53-0.66)	0.58 (0.54-0.68)	ns
Arginine	59.9 (53.5-71.2)	48.5 (38.6-59.5)	0.007
SDMA	0.50 (0.43-0.57)	0.46 (0.43-0.66)	ns
sVCAM-1	1144.5 (989.9-1282.8)	1109.5 (727.2-1577.6)	ns
AVN			
ADMA	0.59 (0.54-0.68)	0.59 (0.53-0.62)	ns
Arginine	54.8 (46.6-66.9)	64.7 (56.3-84.7)	ns

Table 2 | Continued

	without	with	p
SDMA	0.50 (0.44-0.58)	0.42 (0.40-0.50)	ns
sVCAM-1	1126.2 (985.8-1356.6)	1089.4 (711.4-1165.6)	ns
Leg ulcera			
ADMA	0.58 (0.53-0.65)	0.70 (0.68-0.82)	0.012
Arginine	53.9 (46.0-71.0)	59.5 (56.7-61.0)	ns
SDMA	0.49 (0.43-0.56)	0.58 (0.50-0.84)	ns
sVCAM-1	1096.8 (814-1294.2)	1702.6 (1397.5-2374.8)	0.016
Priapism			
ADMA	0.58 (0.53-0.65)	0.79 (0.63-0.86)	0.02
Arginine	54.2 (47.0-65.8)	58.2 (41.6-71.2)	ns
SDMA	0.48 (0.42-0.58)	0.53 (0.46-0.84)	ns
sVCAM-1	1124.2 (814-1294.2)	1288.6 (1008.6-1877.8)	ns
≥2 crises			
ADMA	0.59 (0.53-0.68)	0.63 (0.58-0.65)	ns
Arginine	56.0 (47.0-68.6)	51.4 (48.5-71.2)	ns
SDMA	0.50 (0.44-0.58)	0.41 (0.39-0.44)	ns
sVCAM-1	1120.4 (814-1356.6)	1162.8 (1126.1-1370.2)	ns
Alpha- thalassemia			
ADMA	0.63 (0.54-0.68)	0.58 (0.49-0.60)	ns
Arginine	61.0 (48.5-68.6)	54.4 (41.6-70.9)	ns
SDMA	0.55 (0.48-0.66)	0.45 (0.40-0.55)	0.02
sVCAM-1	1238.6 (1053.6-1356.6)	1040.3 (746.7-1164.2)	ns

ns= not significant

Data for stroke were not applicable

Data are presented as medians with their corresponding interquartile range.

Between group differences were tested with the Mann-Whitney *U* test

DISCUSSION

The current study confirms the close associations of ADMA to the hemolytic phenotype of SCD, as plasma ADMA concentrations were significantly higher in patients with several hemolysis-associated complications next to PHT such as leg ulcers, priapism and cholelithiasis. ADMA concentrations were, however, not associated to complications of the vaso-occlusive phenotype such as painful crises and ACS. The latter is in accordance with our recent finding that plasma ADMA concentrations do not increase further during painful crises.[15] Plasma ADMA levels were lower in patients with α-thalassemia, even though this did not reach statistical significance. Serum LDH levels were higher only in patients with PHT and microalbuminuria (LDH levels would have been expected to be higher in patients with hemolysis associated complications). The relatively low number of patients is the likely explanation for these findings, as both α-thalassemia and LDH are related to hemolysis associated complications in several larger studies.[16,17] The lack of statistically significant relations of arginine/ADMA to hemolysis associated complications may be the result of both the greater variation of ratio's as compared to ADMA and the relatively small patient sample. However, it is unknown at present whether ADMA concentrations or arginine/ADMA ratio's are better predictors of NOS inhibition.

NTproBNP and BNP are natriuretic hormones mainly released into the blood due to cardiomyocyte stretch. Their serum levels reflect the extent of cardiac chamber volume and pressure overload.[18] Both are accepted as diagnostic and prognostic markers for patients with PHT,[19] and several studies have suggested serum (pro)BNP levels to indicate the presence of PHT in SCD.[20,21] It is well established that the major factor determining the development of left ventricular hypertrophy in SCD is the degree of anemia which, before onset of renal dysfunction, is mainly determined by hemolytic rate.[22] In this light we recently reported hemoglobin levels and renal function to be the most important determinants of (NTpro)BNP levels in SCD, and not the presence of (mild) PHT.[23] The stronger association of (NTpro)BNP to SDMA than to ADMA, with the former being completely renally cleared, further supports the concept that in SCD renal function is a major determinant of (NTpro)BNP levels. Given

the fact that PHT and renal dysfunction often coincide in SCD,[24,25] caution is advised when using (NTpro)BNP as a marker for PHT in SCD.

A limitation of this study is the small number of patients. Consequently, several complications (e.g. stroke in the HbSS/HbSβ°-thalassemia group and all but retinopathy, cholelithiasis and α-thalassemia in the HbSC/HbSβ⁺-thalassemia group) did not occur at a sufficient rate to allow statistical analysis. The present data should therefore be interpreted with caution and need reconfirmation in a larger cohort.

Taken together, our data shows for the first time, an association of plasma ADMA concentrations to hemolysis-related complications in SCD other than PHT thereby supporting the important role of ADMA in the pathophysiology of the SCD hemolytic phenotype.

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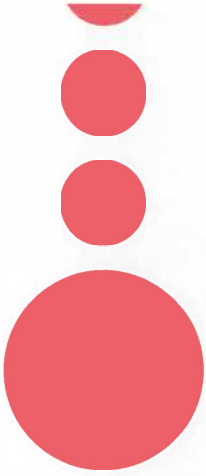

SUMMARY AND CONCLUSION

Sickle cell disease (SCD) is one of the most common hereditary disorders in the world affecting millions of people. SCD is particularly common among those whose ancestors come from Sub-Saharan Africa, Saudi Arabia, India, and Mediterranean countries. The mutation GAG→GTG substitutes valine for glutamic acid at position 6 in the β-globin, gene resulting in the β^S-globin gene. The underlying cause of SCD is the inheritance of two copies of the mutant β^S-globin gene (leading to sickle cell anemia, or the HbSS genotype), or one β^S-globin gene in combination with other abnormal hemoglobin such as HbC or with β-thallasemic genes.

HbS polymerizes upon deoxygenation. The polymers make the erythrocyte rigid, distort its shape, and cause structural damage in the red-cell membrane, all of which alter the rheologic properties of the cell, impair blood flow through the microvasculature, and lead to both hemolysis and vaso-occlusive episodes. The extent of HbS polymerization is proportional to the degree and duration of hemoglobin deoxygenation and intracellular HbS concentration. Fetal hemoglobin in the erythrocyte reduces the concentration of HbS and also reduces its polymerization.

SCD is heterogeneous in its presentation, with differences in the rate and severity of complications even within a single genotype. 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. The complications of SCD are myriad, but the most common is the vaso-occlusive painful crisis. With increasing age and improved supportive care chronic end-organ complications are becoming an increasing challenge for the treating clinician.

The care of patients with SCD has undergone important advances in recent years, with screening programs in many countries leading to timely institution of vaccinations and penicillin prophylaxis as well as the discovery of hydroxycarbamide (also known as hydroxyurea) as an efficacious drug for managing patients with SCD. New drugs are currently being examined. Concepts of individualized pain relief strategies and combination therapy have been further explored in more recent years, also offering promise to improve the care of patients with SCD. The improved supportive care has increased life expectancy of SCD patients in Western countries into the fourth and

fifth decade of life.[1] Nonetheless; increased understanding of the pathophysiology in SCD is needed in order to expand treatment options.

The first chapter of this thesis gives an outline of SCD clinical outcome, pathophysiology and treatment options. As long as the pathophysiology is not completely understood it will be difficult to find effective treatments or treatment strategies. The studies presented in this thesis focus on the potential roles of angiogenesis and NO bioavailability in the pathophysiology of SCD.

Angiogenesis is a well orchestrated process determined by the relative levels of pro-and anti-angiogenic factors involving endothelial cell activation.[2] Angiogenesis is thought to play an important role in the pathophysiology of SCD and SCD-related complications. The formation of abnormal blood vessels and the process of vascular remodeling likely contribute significantly to SCD-related morbidity.[3] SCD patients have been demonstrated to be in a pro-angiogenic state due to increased angiopoietin-2 levels in relation to angiopoietin-1 and vascular endothelial growth factor (VEGF).[4] In chapter two we describe the contribution of anti-angiogenic factors to this pro-angiogenic state in clinically asymptomatic sickle cell patients. SCD is characterized by elevated blood levels of pro-inflammatory cytokines and tissue hypoxia which are both known to increase VEGF and placenta-like growth factor (PlGF) levels.[5] Nevertheless compared to healthy controls no increment of VEGF and PlGF is seen during steady state in SCD which could be related to increased levels of anti-angiogenic factors. Soluble Fms-like tyrosine kinase (sFlt)-1 and soluble endoglin (sEng) are known to sequester and inhibit VEGF, PlGF and transforming growth factor-β1 signaling hence neutralizing their pro-angiogenic response. Compared to healthy controls we found increased sFlt-1 and sEng levels during steady state in our SCD population which are thought to sequester circulating free VEGF/PlGF resulting in unaltered VEGF/PlGF levels.

Endothelial cell activation and dysfunction due to decreased NO bioavailability contribute to angiogenic growth factor release resulting in an impaired ability to mount an adequate angiogenic response which is thought to be implicated in the development of PHT.[6-9] SCD is characterized by continuous endothelial cell activation, ongoing (low grade) tissue ischemia and continuously elevated erythropoietin levels due to the hemolytic anemia, all factors known to induce or contribute to

angiogenesis. In several complications that occur in SCD, such as PHT, retinopathy, *Moyamoya* syndrome and stroke, angiogenesis has proven its role of importance.[3,4,10] Although angiogenic growth factor levels have been shown to differ from healthy controls we did not find a relation between the altered levels and SCD-related complications as shown in chapter three. This does not rule out a role of importance in the development of SCD-related complications since only few angiogenic factors are known which have only recently been studied. This outcome only emphasizes the complexity of the angiogenic response and SCD pathophysiology.

Tissue ischemia is probably one of the leading factors to a pro-angiogenic state that is likely to be involved in reendothelialization and neovascularization.[4] After acute tissue ischemia and endothelial damage, as seen during SCD painful crises, circulating endothelial cells (CECs) are mobilized in order to take part in endothelial neovascularization. CECs are indicative of vascular and endothelial injury.[11] In the study described in chapter four we measured CECs and report a possible relation of *in vivo* CECs to SCD-related organ damage since CEC levels appeared to be highest in those with extensive organ damage.[12]

Tissue damage and hypoxia are both known to up-regulate many angiogenic factors and cytokines such as stromal-derived factor (SDF)-1 and its receptor CXCR4 (CD184) which both have been recognized as key mediators of angiogenesis and inflammation.[13,14] SDF-1 has also been demonstrated to be involved in the recruitment of CECs in order to support the revascularization process after endothelial damage and during hypoxia. Ischemia-reperfusion injury, hemolysis resulting in reduced nitric oxide (NO) and hypoxia are characteristic for SCD therefore we studied the role of SDF-1 in SCD and SCD-related complications in chapter five. No increment of SDF-1 was seen during an uncomplicated painful crisis. However, significant higher levels of SDF-1 have been found in patients with SCD-related pulmonary hypertension (PHT), suggesting a potential role of SDF-1 in SCD-related PHT pathophysiology.

The second part of this thesis focused on the role of the reduced NO bioavailability in the pathophysiology in SCD. NO plays a central role in vascular homeostasis and during the clinically asymptomatic state NO bioavailability is reduced in SCD. Asymmetric dimethylarginine (ADMA) is an endogenous NO synthase inhibitor that

contributes to the chronic NO shortage in SCD. In chapter six we demonstrate that during asymptomatic state ADMA concentrations are elevated and do not show further increment during painful crises. Decreased NO bioavailability due to increased arginase activity and intravascular hemolysis are thought to play a substantial role in the development of SCD-related PHT.[15-17] In chapter seven we show increased ADMA concentration in SCD-related PHT, therefore identifying a possible contributory mechanism leading to reduced NO bioavailability in relation to SCD-related PHT. Chronic hemolysis is considered the driving force behind the NO shortage in SCD.[17,18] Two extremes within the SCD spectrum can be appreciated, i.e. patients with severe hemolysis, PHT, leg ulcers, priapism and stroke, which some refer to as the hemolytic phenotype and those with low rates of hemolysis, high incidence of painful crises and acute chest syndrome, which some refer to as the vaso-occlusive phenotype. Indeed, as demonstrated in chapter eight, ADMA concentrations were significantly higher in patients with higher hemolytic rates.

FUTURE PERSPECTIVES

SCD is a complex and debilitating disease. Since there are little therapeutic options rapid understanding of the pathophysiology is merited also to prevent development of SCD-related complications. Accurate objective laboratory parameters that reflect the *in vivo* rate of developing organ damage in SCD are needed since laboratory markers such as lactate dehydrogenase, leukocyte count and fetal hemoglobin percentage are not sufficiently reliable in predicting developing organ damage in the individual patient.[19] In order to monitor *in vivo* endothelial damage in SCD endothelial cell specific and easily measurable markers are necessary. In SCD biomarkers are thought to play an important role in disease management and many different markers in blood and urine have been described. Nevertheless their value herein is unclear since only few biomarkers relate clearly or causally to pathological processes.[20] Finding suitable biomarkers would facilitate early diagnosis of complications, early detection of (chronic) organ damage and might also be useful in monitoring response to treatment. Biomarkers might even be able to point out high-risk patients in SCD. Due to the complex pathophysiology finding such a marker within the known angiogenic factors is challenging. Anti-angiogenic factors play a role in maintaining the

pro-angiogenic state yet their role in the development of SCD-related complications is still to be elucidated. One might hypothesize that during the development of SCD-related complications the levels of anti-angiogenic factors substantially increase in an attempt of the body to counteract angiogenesis as is seen in pre-eclampsia.[21,22] Prospective studies are necessary in which patients are enrolled from birth. Frequent blood sampling, detailed monthly history and yearly screening for SCD-related complications might provide angiogenic risk profiles enabling early detection of developing complications.

We have shown that CECs might be associated to SCD-related organ damage. Further study should address the factors that are of importance for the mobilization of CECs in SCD as this may reveal potential markers of disease severity.

Recently Parent *et al* have shown by right heart catheterization that the estimated prevalence of SCD-related PHT seems to be 6% instead of 30% as measured by echocardiography.[23] Nevertheless they and earlier studies have shown that the mortality rate still remains high in SCD patients with TRV $\geq$2.5 m/sec. Even more, autopsy studies show signs of PHT in 30% of SCD patients.[24] Given the provocative data recently published by Parent *et al* angiogenic factors should also be studied in SCD patients with PHT confirmed by right heart catheterization.[23] Since ADMA seems to be associated to several nonhemolytic driven forms of PHT and is associated with SCD-related PHT it would be interesting to correlate findings with right heart catheterization to ADMA concentrations.[25]

With further regard to ADMA a recent study showed *in vitro* and *in vivo* that in atherosclerotic disease the use of flavonol quercetin, a product that is found in red wine and tea, decreased the systemic ADMA levels.[26] In a healthy population an increase of NO bioavailability was shown in response to flavonol quercetin.[27] Therefore, the potential effect of flavonol quercetin on ADMA concentrations should also be studied in SCD patients.

As of yet allogeneic stem cell transplantation is the only curative treatment in SCD. Patients eligible for bone marrow transplant are those with severe disease defined by history of stroke, >3 severe vaso-occlusive crises per year, osteonecrosis and red blood cell alloimmunization. Studies have shown a 94% probability of survival and an 84% event free survival.[28,29] Nevertheless due to lack of suitable matched donors and

the chronic use of immunosuppressive drugs with serious side effects the treatment is rarely used. Moreover, most sickle cell patients live in countries where stem cell transplantations are not readily available. Studies on curative treatment are scarce in SCD yet recently a very promising study has been published showing that silencing of BCL11A, which is a repressor of γ-globin expression, in an animal study induced high level fetal hemoglobin induction correcting the hematologic and pathologic defects associated in SCD.[30] Although with this study a potential for an effective or even 'curative' treatment may be found, this is not likely to benefit most patients. In order to keep developing novel widely applicable therapeutics the search to markers that define the pathophysiology in SCD remains of importance in order to enable risk assessment and to optimize therapeutic regiment.

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SAMENVATTING EN CONCLUSIE

Sikkelcelziekte (SCZ) is wereldwijd één van de meest voorkomende erfelijke aandoeningen. Oorspronkelijk werd de ziekte gezien in Afrika, Saudi-Arabië, India en mediterraan gelegen landen echter door slavernij en migratie komt de ziekte nu wereldwijd voor. SCZ wordt veroorzaakt door een puntmutatie op positie 6 in het β-globine gen waardoor valine vervangen wordt door glutaminezuur resulterend in het β^s-globine gen, HbS. De onderliggende oorzaak van SCZ is de overerving van twee kopieën van het mutante β^s-globine gen (wat leidt tot sikkelcelanemie, of het HbSS genotype), of één β^s-globine gen in combinatie met een andere abnormale hemoglobine zoals HbC of β-thallasemie.

Door de mutatie in sikkelcellen vormt de hemoglobine bij deoxygenatie lange ketens van polymeren. De polymeren maken erytrocyten rigide, waarna zij tot vervorming en structurele schade leiden aan de rode bloedcelwand. Dit alles verandert de rheologische eigenschappen van de cel, dat resulteert in een bemoeilijkte bloeddoorstroming door de microvascularisatie, hemolyse en vaso-occlusieve episodes. De mate van HbS polymerisatie wordt beïnvloed door hemoglobine deoxygenatie als ook de intracellulaire HbS en foetaal hemoglobine concentratie.

Binnen één genotype worden er verschillen in de mate en ernst van complicaties gezien. Zelfs de presentatie van patiënten met het ergste genotype, HbSS, kunnen variëren van frequente opnames voor behandeling van acute complicaties tot zelden een noodzaak voor medische interventie. Er zijn veel complicaties beschreven van SCZ maar de meest voorkomende is de vaso-occlusieve pijncrise. Naar mate patiënten ouder worden en er steeds betere ondersteunende behandelingen zijn worden chronische orgaancomplicaties een grotere uitdaging voor de behandelaar.

De zorg van patiënten met SCZ is de afgelopen jaren sterk verbeterd door screeningsprogramma's wat in veel landen leidt tot tijdige vaccinering en penicilline profylaxe. Ook de ontdekking van hydroxycarbamide (beter bekend als hydroxyurea) is een effectief/doeltreffend middel voor de behandeling van SCZ. Concepten van individuele pijnbestrijdingstrategieën en combinatietherapie lijken veelbelovende therapieën te zijn die de laatste jaren worden onderzocht ter verbetering van de zorg voor SCZ. De levensverwachting van patiënten met SCZ in Westerse landen is

door verbeterde medische zorg gestegen naar een gemiddelde levensverwachting van veertig à vijftig jaar.[1] Echter, aangezien in de Westerse landen de gemiddelde levensverwachting rond de tachtig jaar ligt is beter begrip van de pathofysiologie in SCZ nodig om de behandelopties te vergroten en de levensverwachting nog verder op te trekken richting die van de gemiddelde populatie.

Het eerste hoofdstuk van dit proefschrift geeft een overzicht van de klinische uitkomsten, pathofysiologie en huidige behandelopties. De overige hoofdstukken in dit proefschrift richten zich op de potentiële rol van angiogenese en de biologische beschikbaarheid van nitriet oxide (NO) in de pathofysiologie van SCZ.

Angiogenese is een goed georganiseerd proces dat beïnvloed wordt door de relatieve waarden van de pro-en anti-angiogene factoren.[2] Van angiogenese wordt gedacht dat het een belangrijke rol speelt in de pathofysiologie van SCZ en aan SCZ-gerelateerde complicaties. De vorming van abnormale bloedvaten en het proces van vasculaire remodellering dragen waarschijnlijk significant bij aan de morbiditeit.[3] In de studie van Duits *et al* wordt aangetoond dat patiënten met SCZ in een pro-angiogene staat zijn wat ten gevolge van gestegen angiopoietine-2 waarden in relatie tot angiopoietine-1 en vascular endothelial growth factor (VEGF) waarden is.[4] In hoofdstuk twee beschrijven we de bijdrage van anti-angiogene factoren aan de in standhouding van de pro-angiogene staat bij klinisch asymptomatische SCZ patiënten. Verhoogde waarden van pro-inflammatoire cytokinen en weefsel hypoxie dragen bij aan een stijging van VEGF en placenta-like growth factor (PlGF) waarden.[5] Desondanks wordt er, vergeleken met gezonde mensen, geen stijging van VEGF en PlGF gezien tijdens de asymptomatische staat in SCZ wat een gevolg lijkt te zijn van gestegen anti-angiogene factor waarden. Soluble Fms-like tyrosine kinase (sFlt)-1 en soluble endoglin (sEng) sekwestreren en inhiberen signalen van VEGF, PlGF en transforming growth factor-β1, daarmee de pro-angiogene respons neutraliserend. Vergeleken met gezonden werden verhoogde sFlt-1 en sEng waarden gemeten in de asymptomatische sikkelcel populatie. De onveranderde VEGF/PlGF waarden ontstaat doordat sFlt-1 en sEng vrij VEGF/PlGF wegvangen.

Endotheliale cel activiteit en disfunctie ten gevolge van verminderde NO biobeschikbaarheid dragen bij aan het vrijkomen van angiogene groei factoren. Dit resulteert in een veranderd vermogen om een adequate angiogene respons te

bewerkstelligen die van belang geacht wordt bij de vorming cq het ontstaan van pulmonale hypertensie (PHT).[6-9] SCZ wordt verder ook gekarakteriseerd door factoren die angiogenese induceren of hieraan bijdragen zoals continue endotheliale activatie, continue (laaggradige) weefsel ischemie en continue geëleveerde erytropoietine waarden ten gevolge de hemolyse. In sommige complicaties die bij SCZ voorkomen, zoals PHT, retinopathie, *Moyamoya* syndroom en beroerte is de rol van angiogenese beschreven.[3,4,10] Alhoewel in SCZ angiogene groeifactor waarden blijken te verschillen van gezonde mensen wordt in hoofdstuk zes geen relatie gevonden tussen veranderde angiogene groeifactor waarden en SCZ-gerelateerde complicaties. Dit sluit een rol voor angiogene factoren in de ontwikkeling van SCZ-gerelateerde complicaties niet uit aangezien slechts enkele angiogene factoren bekend zijn en zij pas sinds kort onderzocht worden. Deze uitkomst benadrukt juist de complexiteit van de angiogene respons en pathofysiologie van SCZ.

Weefsel ischemie is waarschijnlijk een van de voornaamste factoren in de pro-angiogene staat die betrokken lijkt te zijn bij de reendothelialisatie en neovascularisatie.[4] Na acute weefsel ischemie en endotheliale schade, zoals gezien wordt tijdens SCZ pijncrisen, worden circulerende endotheliale cellen (CECs) gemobiliseerd om deel te nemen aan de endotheliale neovascularisatie. CECs zijn indicatief voor vasculaire en endotheliale schade.[11] In hoofdstuk drie worden de CEC waarden gemeten.[12] Zij geven een mogelijke relatie weer van *in vivo* CECs ten aanzien van SCZ-gerelateerde orgaanschade daar CEC waarden het hoogst zijn bij die genen die de ergste vorm van orgaanschade vertonen.

Weefselschade en hypoxie staan er bekend om angiogene factoren en cytokinen op te reguleren zoals stromal-derived factor (SDF)-1 en zijn receptor CXCR4 (CD184). SDF-1 en zijn receptor worden op hun beurt gezien als belangrijke mediatoren van angiogenese en inflammatie.[13,14] Van SDF-1 is ook aangetoond dat zij betrokken is bij het aantrekken van CECs om het revascularisatie proces te ondersteunen na endotheliale schade en tijdens weefsel hypoxie. Ischemie-reperfusie schade, hypoxie en hemolyse resulterend in verlaagde NO biobeschikbaarheid zijn karakteristiek voor SCZ. In hoofdstuk vijf wordt de rol van SDF-1 in SCZ en SCZ-gerelateerde complicaties beschreven. Er werd geen toename van SDF-1 gezien tijdens ongecompliceerde pijncrise. Significant hogere waarden van SDF-1 zijn gezien in patiënten met

SCZ-gerelateerde PHT, wat kan duiden op een potentiële rol voor SDF-1 in de pathofysiologie van SCZ-gerelateerde PHT.

Het tweede deel van dit proefschrift richt zich op de rol van de NO biobeschikbaarheid in de pathofysiologie van SCZ. NO speelt een centrale rol in de vasculaire homeostase en tijdens de klinische asymptomatische staat is NO biobeschikbaarheid gereduceerd. Asymmetrische dimethylarginine (ADMA) is een endogene NO synthase inhibitor die bijdraagt aan het chronische NO tekort in SCZ. In hoofdstuk zeven wordt aangetoond dat tijdens de asymptomatische staat ADMA concentraties verhoogd zijn; deze waarden stijgen echter niet verder tijdens een pijncrise. Van verlaagde NO biobeschikbaarheid ten gevolge van toegenomen arginase activiteit en intravasculaire hemolyse wordt gedacht dat zij een substantiële rol spelen in de ontwikkeling van SCZ-gerelateerde PHT.[15-17] In hoofdstuk acht worden de gestegen ADMA concentraties in SCZ-gerelateerde PHT getoond, hiermee een mogelijke bijdragend mechanisme identificerend tot gereduceerde NO biobeschikbaarheid in relatie tot SCZ-gerelateerde PHT. Chronische hemolyse wordt gezien als de drijvende kracht achter het NO tekort in SCZ.[17,18] Binnen het SCZ spectrum worden twee extremen gezien. Dit zijn patiënten met ernstige hemolyse, PHT, beenulcera, priapisme en beroerten wat door sommigen omschreven wordt als het hemolytische fenotype versus die genen met lage mate van hemolyse, hoge incidentie van pijncrise en acuut chest syndroom wat door sommigen gezien wordt als het vaso-occlusieve fenotype. Zoals aangetoond in hoofdstuk negen zijn de ADMA waarden significant hoger in patiënten met hogere hemolytische waarden.

TOEKOMSTPERSPECTIEVEN

SCZ is een complexe ziekte met een hoog morbiditeitgehalte. Aangezien er weinig therapeutische opties zijn is snel begrip van de pathofysiologie noodzakelijk om de ontwikkeling van SCZ-gerelateerde complicatie te voorkomen. Accurate objectieve laboratorium parameters die *in vivo* de mate van ontwikkeling van orgaanschade in SCZ reflecteren zijn nodig aangezien biomarkers zoals lactaat dehydrogenase, leukocyten aantal en het foetale hemoglobine percentage onvoldoende betrouwbaar zijn in het voorspellen van de ontwikkeling van orgaanschade in de individuele patiënt.[19] Om de *in vivo* endotheliale schade te monitoren in SCZ zijn endotheliale cel specifieke en

makkelijk te meten markers nodig. In SCZ wordt een belangrijke rol toegeschreven aan biomarkers voor ziekte management. Zowel in bloed als urine zijn markers beschreven, desondanks is hun waarde onduidelijk aangezien er van slechts enkele biomarkers een duidelijk causaal verband met pathologische processen gevonden is.[20] Het vinden van geschikte biomarkers zou vroege detectie van (chronische) orgaanschade vergemakkelijken wat ook van waarde is tijdens het monitoren van respons op behandeling. Biomarkers zouden zelfs in staat kunnen zijn om hoogrisico patiënten aan te tonen in SCZ. Ten gevolge van de complexe pathofysiologie is het vinden van zo een marker een grote uitdaging. Anti-angiogene factoren spelen een rol in het handhaven van de pro-angiogene staat echter de rol in de ontwikkeling van SCZ-gerelateerde complicaties dient nog aangetoond te worden. Hypothetisch gezien zou het zo kunnen zijn dat tijdens de ontwikkeling van SCZ-gerelateerde complicaties de waarden van anti-angiogene factoren substantieel stijgt in een poging van het lichaam om angiogenese tegen te werken zoals gezien wordt bij pre-eclampsie.[21,22] Prospectieve studies zijn nodig waarbij patiënten vanaf de geboorte geïncludeerd worden. Frequente bloedafnames, gedetailleerde maandelijkse anamnese en jaarlijkse screening voor SCZ-gerelateerde complicaties kunnen wellicht angiogene profielen tentoonspreiden die vroege detectie van ontwikkeling van complicaties kan bewerkstelligen.

In dit proefschrift wordt een mogelijke associatie aangetoond tussen CECs en SCZ-gerelateerde orgaanschade. Verdere onderzoeken zijn nodig naar de factoren die van belang zijn voor de mobilisatie van de CECs in SCZ aangezien zij potentiële biomarkers voor ziekte ernst zouden kunnen ontwaren.

Recent heeft Parent *et al* aangetoond door middel van rechtszijdige hartkatheterisatie de geschatte prevalentie van SCZ-gerelateerde PHT 6% blijkt te zijn in plaats van 30% die aangetoond wordt door echocardiografie. Desondanks tonen zij en anderen een hoge mortaliteit aan in sikkelcel patiënten met tricuspide regurgitatie jet flow van $\geq$2.5 m/sec. Daarnaast tonen autopsie studies in 30% van de sikkelcel patiënten aanwijzingen voor PHT.[24] Gezien de provocerende data gepubliceerd door Parent *et al* zouden angiogene factoren ook bestudeerd moeten worden in SCZ patiënten met PHT aangetoond met rechtszijdige hartkatheterisatie. Aangezien ADMA geassocieerd is met verschillende vormen van PHT die niet door hemolyse gedreven

worden en is geassocieerd met SCZ-gerelateerde PHT is het interessant om de uitkomsten te correleren aan rechtszijdige hartkatheterisatie en ADMA concentraties.

Ten aanzien van ADMA heeft recent onderzoek *in vitro* en *in vivo* aangetoond dat bij atherosclerose gerelateerde ziekten flavonol quercetin, een product dat gevonden wordt in rode wijn en thee, de systemische ADMA concentratie reduceert.[26] In een gezonde populatie werd een toename van de NO biobeschikbaarheid gezien in respons op het flavonol quercetin. Daarom dient het potentiële effect van flavonol quercetin op ADMA concentraties ook in de sikkelcel populatie bestudeerd te worden.

Allogene stamceltransplantatie is de enige curatieve behandeling voor SCZ. Patiënten kunnen in aanmerking komen voor een beenmergtransplantatie als zij een ernstige ziektevorm hebben welke gedefinieerd wordt door een voorgeschiedenis met waarin beroerte, ≥3 ernstige vaso-occlusieve crisen per jaar, osteonecrose en rode bloedcel alloimmunizatie. Studies hebben aangetoond dat er een 94% overlevingskans bestaat en 84% kans op een complicatie vrije overleving.[28,29] Door het tekort aan geschikte donoren en het chronische gebruik van immunosuppressiva met ernstige bijwerkingen wordt de behandeling zelden gebruikt. Verder leven de meeste sikkelcel patiënten in landen waar de stamceltransplantatie niet gemakkelijk voor handen is. Onderzoeken naar curatieve behandeling zijn zeldzaam binnen SCZ echter recent is er een veelbelovende studie gepubliceerd die gebruik maakt van silencing van BCL11A. BCL11A onderdrukt de γ-globine expressie, in een dierenstudie wordt een hoge concentratie foetaal hemoglobine geïnduceerd wat de hematologische en pathologische defecten in SCZ lijkt te corrigeren.[30] Hoewel met deze studie een potentieel effectieve of zelfs "curatieve" behandeling gevonden lijkt te zijn, zullen niet veel patiënten hier profijt van hebben aangezien het merendeel van de patiënten in de zogenaamde 3de wereldlanden woonachtig is. Om wereldwijd toepasbare therapieën te ontwikkelen is de zoektocht naar markers die de pathofysiologie van SCZ definiëren van belang ten faveure van risicostratificatie en optimalisatie van therapeutische regimenten.

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THE CURAMA STUDY GROUP

The CURAMA study group is a collaborative effort studying sickle cell disease in the Netherlands Antilles and the Netherlands. The participating centers are: The Red Cross Blood Bank Foundation, Curaçao, Netherlands Antilles; The Antillean Institute for Health Research, Curaçao, Netherlands Antilles, The Department of Internal Medicine, Slotervaart Hospital, Amsterdam, The Netherlands; the Department of Vascular Medicine and the Department of Hematology, Academic Medical Center, Amsterdam, The Netherlands; the Department of Hematology, Erasmus Medical Center, Rotterdam, The Netherlands; the Department of Pathology, Groningen University Hospital, The Netherlands; the Department of Internal Medicine, Laboratory of Clinical Thrombosis and Hemostasis, and the Cardiovascular Research Institute, Academic Hospital Maastricht, The Netherlands.

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LIST OF PUBLICATIONS

Landburg PP, Teerlink T, Muskiet FA, Duits AJ, Schnog JJ; CURAMA study group. Plasma concentrations of asymmetric dimethylarginine, an endogenous nitric oxide synthase inhibitor, are elevated in sickle cell patients but do not increase further during painful crisis. ***Am J Hematol. 2008 Jul;83(7):577-9.***

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Curriculum Vitae

Precious Pearl Landburg was born in 1980 in Amsterdam, the Netherlands. She is the daughter and only child of Carmelita Landburg-Wellis and Roberto Landburg, who are originally from Surinam, South America. After finishing secondary school at the Gooisch Lyceum in Bussum, she entered medical school at the Erasmus University Rotterdam in 1998. Her senior internship internal medicine was on Curaçao, which is an island in the Caribbean. Here she also started to work as a junior medical doctor after obtaining her medical degree. On Curaçao her interest in research increased resulting in the projects which are described in this thesis under the supervision of Prof. dr. A.J. Duits and dr. J.B Schnog. During this period she became an ACLS provider, she was a member of the resuscitation board (and team) of the Sint Elisabeth Hospital and she also participated in the development of the annually held Critical Care and Emergency Conference on Curaçao which has the Nederlands-Caribische Stichting voor Klinisch Hoger Onderwijs (NASKHO) and the Massachusetts General Hospital, Boston, USA as conference partners. In 2009 she started her Internal Medicine residency in the Slotervaartziekenhuis followed by the Onze Lieve Vrouwe Gasthuis both sided in Amsterdam. In October 2012 she started with a two-year fellowship Intensive Care medicine in the Academic Medical Centre of Amsterdam.