Vasculopathy and Pulmonary Hypertension in Sickle Cell Disease

Karin P. Potoka 1,2 and Mark T. Gladwin1,3*

Affiliations:
1Pittsburgh Heart, Lung, Blood and Vascular Medicine Institute, University of Pittsburgh.
2Division of Newborn Medicine, Department of Pediatrics, University of Pittsburgh.
3Division of Pulmonary, Allergy and Critical Care Medicine, University of Pittsburgh.

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Reprint requests to Dr. Gladwin at Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh, NW 160 Montefiore Hospital, 354 Fifth Avenue, Pittsburgh, PA, 15213, or at gladwinmt@upmc.edu.
SICKLE CELL DISEASE is an autosomal recessive disorder in the gene encoding the $\beta$-chain of hemoglobin. Deoxygenation, causes the mutant hemoglobin S to polymerize resulting in rigid, adherent red cells that are entrapped in the microcirculation and hemolyze. Cardinal features include severe painful crises and episodic acute lung injury, called acute chest syndrome. This population, with age, develops chronic organ injury, such as chronic kidney disease and pulmonary hypertension. A major risk factor for developing chronic organ injury is hemolytic anemia, which releases red cell contents into the circulation. Cell free plasma hemoglobin, heme, and arginase 1 disrupt endothelial function, drive oxidative and inflammatory stress, and have recently been referred to as erythrocyte damage associated molecular pattern molecules (eDAMPs). Studies suggest that in addition to effects of cell free plasma hemoglobin on scavenging nitric oxide (NO) and generating reactive oxygen species (ROS), heme released from plasma hemoglobin can bind to the toll-like receptor 4 to activate the innate immune system. Persistent intravascular hemolysis over decades leads to chronic vasculopathy, with about 10% of patients developing pulmonary hypertension. Progressive obstruction of small pulmonary arterioles, increase in pulmonary vascular resistance, decreased cardiac output and eventual right heart failure causes death in many patients with this complication. This review provides an overview of the pathobiology of hemolysis-mediated endothelial dysfunction and eDAMPs and a summary of our current understanding of diagnosis and management of pulmonary hypertension in sickle cell disease, including a review of recent ATS consensus guidelines for risk stratification and management.
Introduction

Sickle cell disease is an autosomal recessive disease caused by point mutations in the gene that encodes the β-globin chains of hemoglobin. There are two alleles coding for β-globin and two β-globin molecules combine with two α-globins (encoded by 4 alleles) to form the normal hemoglobin tetramer. In approximately 75% of patients with sickle cell disease, a single nucleotide substitution (A to T) in the codon for amino acid 6 causes a substitution of glutamine for a valine producing hemoglobin S (HbS). The valine is only exposed in the deoxygenated T-state tetramer and produces a contact site between β-chains leading to polymerization of the hemoglobin in deoxygenated erythrocytes. The other common form of sickle cell disease is HbSC disease, caused by dual mutations in the two β-globin alleles, one coding for the glutamic acid to valine substitution and the other for a valine to lysine substitution. Other compound heterozygote inheritance patterns can lead to sickle cell disease, such as HbS-beta thalassemic mutations, where the thalassemia mutation reduces the expression of a normal β-globin protein and therefore enhances the relative expression of the mutant β-sickle globin and HbS.

Of relevance to disease pathogenesis and treatment, hemoglobin comes in various forms, which are expressed at different stages of development in fetuses, infants and adults. In early fetal life until about 6-months of age γ-globin is expressed instead of the β-globin and combines with α-globins to form fetal hemoglobin (HbF) tetramers. Because the HbF hemoglobin does not contain the HbS mutation, sickle cell disease does not become manifest until about 6 months of life, with fevers, irritability, poor food intake, splenomegaly and swelling and inflammation of the fingers, called dactylitis. Genetic variability in adult HbF expression explains much of the disease phenotypic variability, with patients with high HbF levels, often resulting from reduced expression of the transcriptional repressor BCL11A, exhibiting milder disease severity.(12, 61, 88) The FDA approved medication hydroxyurea acts by increasing the expression of HbF in children and adults.

The pathogenesis of sickle cell disease is driven by the principles of hemoglobin S polymerization within red blood cells. This is a biophysical process obeying liquid crystallization kinetics, with the extent of HbS polymerization proportional to the degree of hemoglobin deoxygenation, time, and the concentration of HbS to the $15^{th}$ power (9). From a clinical standpoint, factors that modulate these factors will increase red cell HbS polymerization and cause flares of disease. These factors include hypoxia and
high pH (reducing HbS oxygen affinity), lag time of red cells in the microcirculation caused by inflammation and cellular adhesive events, and an increase in the intra-erythrocytic HbS concentration, often mediated by the activation of membrane ion channels like the Gardos channel that deplete intracellular water. Red blood cells with high HbS polymer content become rigid and become entrapped in the microcirculation, leading to ischemia and reperfusion events. This is amplified by primary or secondary inflammation, which increases the expression of critical adhesion molecules on red cells, endothelium, leukocytes and platelets that lead to cellular aggregates in the post-capillary venules. This process is caused vaso-occlusive painful crisis and leads to severe tissue ischemia, reperfusion injury and infarction of virtually any organ system. Bone marrow and periosteal infarction results in severe pain. Vaso-occlusion in the brain leads to stroke, in the lung leads to a lung injury syndrome called the acute chest syndrome, and in the spleen, kidney, heart, and skeletal muscle leads to organ-specific dysfunction. We direct the reviewer to recent comprehensive reviews that summarize in more detail the genetics, epidemiology and clinical manifestations of this complex and common monogenetic disease.(19, 39, 77)

Intracellular HbS polymerization also leads to red blood cell hemolysis, secondary to mechanical shearing of the red cell membrane by the intracellular HbS crystal polymers and also secondary to intracellular oxidative stress and metabolic stress which reduce membrane fluidity and increase the expression of surface phosphatidyl serine residues. Hemolysis causes chronic anemia with a compensatory high cardiac output state, necessary to sustain oxygen delivery, and hyperbilirubinemia with an increase in the formation of bilirubin gallstones and causing jaundice. It is now appreciated that intravascular hemolysis also releases toxic red blood cell products that impair endothelial function and drive oxidative and inflammatory stress, ultimately leading to chronic vasculopathy and pulmonary hypertension.(37, 39, 45, 59, 64, 79, 82) In the current review we will focus on this aspect of sickle cell disease pathogenesis, with a summary of the mechanisms and effects of hemolysis on vascular function and a review of the risk factors, prevalence and impact of pulmonary hypertension in patients with sickle cell disease. We will also summarize new therapies targeting sickle cell vasculopathy and available animal models used to study sickle cell lung disease.

Hemolytic Anemia, Erythrocyte-DAMPS and Vasculopathy
Although the spleen is the natural site of RBC removal from the circulation, this system is impaired in patients with SCD. Over time, patient's spleens are subject to recurrent vaso-occlusion and infarction, resulting in auto-splenectomy. This functional asplenia perpetuates and likely increases the rates of intravascular hemolysis with the sustained release of red blood cell microparticles and intracellular contents into the circulation. From a clinical perspective, it is increasingly clear that the severity of hemolytic anemia, as measured directly by products of intravascular hemolysis (cell free plasma hemoglobin and red cell microparticles) and by indirect measures (low plasma haptoglobin levels, reticulocyte counts, bilirubin levels, red cell aspartate aminotransferase and lactate dehydrogenase enzymes) is associated with increased risk of certain vascular complications of sickle cell disease.(68) These include low oxygen saturations and pulmonary hypertension, increased systemic systolic artery pressures and pulse pressures, chronic kidney disease and proteinuria, cutaneous leg ulcerations, and stroke in children. In numerous clinical cohorts the severity of both anemia and measured indices of hemolytic rate are risk factors for developing pulmonary hypertension in sickle cell patients.(6, 23, 36, 37, 40, 46, 48, 49, 54, 63) Interestingly, this is in contrast to the risk factors for other major complications, such as vaso-occlusive painful crisis and the acute chest syndrome, which are associated with higher steady state hemoglobin levels (lower hemolysis) and high leukocyte counts (more inflammation).(73) These associations suggest the existence of two major disease mechanisms that underlie sickle cell disease pathogenesis and lead to discrete phenotypes: 1) hemolysis-endothelial dysfunction leading to vasculopathy, and 2) inflammation and hyperviscosity leading to vaso-occlusion.(39) While the epidemiology links risk of disease phenotype to these mechanisms, most patients do not present at the extreme end of the continuum and significant overlapping features exist. In addition, during acute vaso-occlusive crisis the red blood cells can hyperhemolyze, likely leading to the activation of both mechanisms during acute severe disease exacerbations.

There are a number of mechanisms of hemolysis that lead to vasculopathy and are caused by the release of molecules that are typically compartmentalized within the red blood cell, which shields the endothelium from their pathological effects. With increased studies characterizing the pathogenic properties of these intracellular molecules when released into the plasma, these have now been collectively referred to as erythrocyte danger associated molecular pattern molecules (eDAMPs), which similar to other intracellular DAMPs, are released during massive tissue injury and can activate innate
immunity pathways via the toll-like receptors (TLRs) and the NOD-like receptors of the inflammasome.(35)

Three major effects of red blood cell hemolysis have been characterized that can lead to vascular dysfunction, injury and inflammation (Figure A):

1) Alteration of the balance between nitric oxide (NO) and reactive oxygen species (ROS) leading to impaired endothelial function. The red blood cell releases significant concentrations of hemoglobin into the plasma when lysed. Hemoglobin is a potent NO scavenger via the NO dioxygenation reaction and iron-nitrosylation reactions.(69) As little as 6microM cell free plasma hemoglobin completely impairs NO signaling in endothelium.(25) The levels of cell free hemoglobin in patients with sickle cell disease are tightly correlated to an intrinsic resistance to NO signaling, as measured experimentally by impaired blood flow responses to infusions of NO donor medications, in both humans(78) and transgenic mouse models of sickle cell disease.(44, 51, 52, 66) In addition to plasma hemoglobin, the red blood cell contains significant concentrations of the enzyme arginase 1, which can metabolize L-arginine to ornithine. This reduces L-arginine availability, which is required for de novo NO synthesis by endothelial NO synthase enzyme. Both arginase 1 enzyme activity and the ratio of the levels of arginine/ornithine are reduced in patients with sickle cell disease with higher rates of hemolysis, and predict both the development of pulmonary hypertension and the risk of death.(64)

In addition to inhibition of NO, cell free hemoglobin can redox cycle and undergo fenton and peroxidase reactions to form ROS, which further promote endothelial dysfunction. A number of studies have suggested that hemoglobin deposition in tissues, such as the kidney, will oxidize to form ferric and ferryl hemoglobin species that can react with lipids to generate injurious lipid peroxide radicals. This has been clearly demonstrated in the case of myoglobinuria during skeletal muscle injury. (16, 76) Hemoglobinuria has been shown to activate a cascade of compensatory antioxidant and stress responses in the kidney, including a strong induction of the heme metabolizing enzyme hemeoxygenase-1. (8) It is likely that the formation of ROS by cell free hemoglobin activates down-stream oxidases, such as xanthine oxidase and NADPH oxidases that promote vascular oxidative stress. (2, 3, 91)

2) Alteration of the balance between nitric oxide (NO) and reactive oxygen species (ROS) leading to platelet activation, hemostatic activation and oxidative burden. The inhibition of NO
signaling by cell free plasma hemoglobin in platelets also leads to platelet activation and activation of the hemostatic system. (4, 5, 74, 89) In addition, red blood cells contain high levels of ADP, which can activate platelets via the P2Y receptors. (43) Plasma free hemoglobin at the level similar to what is observed in SCD patients, approximately 5 μM, causes extravascular macrophage and neutrophil localization within the lung parenchyma. It also has the ability to activate the endothelium and increase proinflammatory effects. VCAM-1 levels have been show to correlate with both the rate of intravascular hemolysis and with in vivo measures of endothelial dysfunction. (51, 38, 47) Buehler and colleagues infused hemoglobin into a rat model and found a modest increase in plasma oxidative stress markers (malondialdehyde and 4-hydroxynonenal) and increased lung ICAM protein expression, suggesting an oxidative and inflammatory effect. (17) Cell free hemoglobin redox cycling from the oxidized ferric to reduced ferrous hemoglobin occurs at a slower rate, due to the oxidative environment seen in intravascular hemolysis or hemoglobin infusion. (17)

3) **Heme and ATP mediated activation of the innate immune system leading to sterile inflammation.** Recent studies have shown that intravascular oxidation of cell free plasma hemoglobin generates heme in plasma. Heme has been found to activate the TLR4 receptor and downstream NFκB-mediated inflammation. (13, 33) Heme has also recently been shown to increase ROS and induce neutrophils to release DNA NETS, (22) as well as stimulate the release of angiogenic placenta growth factor, which is drives pulmonary vascular cellular proliferation. (90) Interestingly, a high rate of hemolysis is also associated with a high rate of red cell production. New red cells release their nuclei and present significant DNA for metabolism, leading to increasing concentrations of uric acid. Uric acid crystals can bind to the nucleotide-binding oligomerization domain–like receptors (NOD-like receptors) to potentially activate the NALP3 inflammasome and increase IL-1β production. (57) ATP, released during hemolysis will also activate this pathway. Hemolysis-dependent elaboration of heme, ATP and uric acid will all drive sterile inflammation and can be considered a new form of danger associated molecular pattern molecules (eDAMPs). (35)

*Figure A* summarizes these pathways in the vasculature and how they inhibit NO signaling in the vasculature and activate the hemostatic system.

**Pulmonary Vascular Disease in SCD**
Pulmonary Hypertension (PH) is a disease in which the blood pressure within the pulmonary system is elevated. It is characterized by endothelial dysfunction and imbalance between vasodilators and vasoconstrictors within the pulmonary circulation, followed by progressive smooth muscle and neointimal hyperplasia and physical obliteration of the arteriolar circulation. Progressive increases in pulmonary vascular resistance (PVR) lead to right ventricle (RV) failure and reduced cardiac output. Most of the symptoms and signs of PH relate to this decrease in cardiac output, especially with exertion. Symptoms include dyspnea on exertion, pre-syncope and syncope, and peripheral edema with weight gain. The diagnostic evaluation of a patient with suspected PH includes a history, physical examination, Doppler-echocardiography, CAT scan and ventilation-perfusion imaging to exclude thromboembolic disease and advanced lung disease, laboratory screenings, functional assessments of exercise tolerance such as the six-minute walk distance, and right heart catheterization for definitive diagnosis. A distinction between the classifications of PH requires right heart catheterization (RHC), and is required for the diagnosis of PAH and for approval of specific therapy (27). When the mean pulmonary arterial pressure (mPAP) is \( \geq 25 \text{mmHg} \), this is considered PH. To further distinguish if the disease is pre-capillary or post-capillary the left ventricular end-diastolic pressure must be estimated with an indirect assessment of the pulmonary artery occlusion pressure (PAOP) or directly with left heart catheterization. If the “wedge” pressure or pulmonary artery occlusion pressure (PAOP) is \( \leq 15 \text{mmHg} \) this is defined as precapillary disease and termed pulmonary arterial hypertension (PAH), and often classified as Group 1 PAH (27). If the PAOP is \( >15 \text{mmHg} \) it is considered post-capillary pulmonary venous hypertension (PVH) or Group 2 PH.

PH has become increasingly recognized as a chronic complication of SCD and other hemolytic anemias. Using Doppler-echocardiographic screening, up to 30% of adults with SCD have borderline elevations of their estimated pulmonary artery systolic pressures (10,16). Transthoracic echocardiography (Echo) is an efficient tool to estimate elevation in pulmonary artery systolic pressure by measuring the tricuspid regurgitant jet velocity (TRV) and using the modified Bernoulli equation \( P = 4 \times V^2 + \text{right atrial pressure} \). A value of 2.5-2.9 m/sec is considered borderline and is about 2-3 standard deviations above the normal population mean. Despite the fact that only 25% of these patients will have PH confirmed by right heart catheterization, this borderline group remains at high risk of death and for progression to frank PH. Patients with borderline values for TRV who also have a low exercise capacity (six-minute walk
distance of less than 330 meters or who have a high plasma level of N-terminal pro-natriuretic peptide, a pre-pro-hormone released from the cardiomyocyte under pressure stress, are at higher risk of having true PH diagnosed at right heart catheterization. A value of >2.9 m/sec suggests a 67-75% risk of having PH identified at right heart catheterization and is associated with a greater than 10-fold increased risk of death in multiple studies. Additionally, the presence of right heart dilation or a pericardial effusion on Echo is associated with a higher probability of having PH at right heart catheterization and more severe PH with increased mortality (10). The use of TRV alone does not equate to a clinical definition of PH, which requires a right heart catheterization, but it does define mortality risk and identifies patients at higher risk of having PH (16) (38).

Three major studies have screened patients with sickle cell disease for PH and then have confirmed with right heart catheterization. These studies indicate that 6-10% of adult SCD patient population has a mean pulmonary artery pressure ≥ 25 mm Hg, defining PH (12, 31, 38). About half of these patients have a component of diastolic LV dysfunction indicated by the finding of PAOP >15mmHg (post-capillary PH or pulmonary venous hypertension) and half have PAH. Interestingly, even in patients with an elevated PAOP the transpulmonary gradient (mean pulmonary artery pressure minus the PAOP) and the PVR define the risk of death, suggesting that pulmonary vascular disease defines risk of death in this population.(58, 59) Patients diagnosed with PH by RHC with PAH, evident by elevated mPAP, PVR and transpulmonary gradient, have an associated higher mortality rate compared with those diagnosed as post-capillary PH (30, 31). Consistent with a role for hemolytic anemia in the pathogenesis, SCD patients diagnosed with PAH have increased severity of their hemolytic anemia as indicated by lower hematocrit, higher reticulocyte counts, higher lactate dehydrogenase, higher red cell aspartate aminotransferase, and higher bilirubin levels (31, 36). In addition, they have poorer functional capacity evidenced by reduced six-minute walk distance and higher levels of N-terminal pro-brain natriuretic peptide (NT-BNP) (38). Other risk factors independent of hemolytic anemia have also been identified, including markers of iron overload, cholestatic liver dysfunction, systolic systemic hypertension, and renal insufficiency and proteinuria.(37, 41, 85) It is likely that functional asplenia and vascular thrombosis also contribute. Clinical evidence of vascular dysfunction and PH include lower oxygen saturations, dyspnea, and reduced six-minute walk distance (36, 38). In addition to age, these clinical characteristics can be used to as tools in order to better determine the need for screening in patients with SCD.
Summary of recent guidelines for screening and treatment

A consensus guideline has recently been published by the American Thoracic Society and endorsed by the Pulmonary Hypertension Association and the American College of Chest Physicians.(53) These guidelines summarize that an increased risk for mortality is associated with increased TRV ≥ 2.5m/sec, right heart catheterization confirmation of PH with a mean pulmonary artery pressure ≥ 25mmHg (either PAH or PVH), and an elevated NT-BNP ≥ 160pg/mL. Stable SCD patients should be screened with Echo and/or NT-BNP every 1-3 years to assess this mortality risk. If patients are found to have TRV > 2.5m/sec and or NT-BNP > 16-pg/mL suggesting an increased risk of PH and higher mortality, they should undergo right heart catheterization to confirm the diagnosis of PH and guide specific therapy.(29). Patients identified at high risk of death should be screened for co-morbid factors that are treatable, including low oxygen, iron overload, and thrombo-embolic disease. The underlying sickle cell disease should be treated more aggressively as well, with initiation of hydroxyurea therapy or consideration of chronic transfusion therapy. Patients diagnosed with PAH by right heart catheterization are at the highest risk and should be referred to PAH specialists for consideration of FDA-approved PAH therapies (summarized below).

Mouse Models of Sickle Cell Disease

A number of transgenic humanized mouse models of SCD have been developed to better understand the pathophysiology of the disease and evaluate potential therapies. In the early nineties, mouse models of SCD were developed by transgenic expression of sickle human hemoglobin in mice still expressing mouse hemoglobin or bred with beta-thalassemic mice to reduced mouse β-globin levels. However, these mice failed to fully model sickle cell disease phenotype due to the persistent expression of murine adult hemoglobin. To overcome this problem, knockout mice for both adult mouse β-globin genes and both adult mouse α-globin genes were generated, through targeted deletion. The double knockout mice provided the adequate genetic background for mice expressing only human hemoglobin in mature red blood cells. In 1997, the Berkeley (BERK) mouse was generated by developing transgenic mice expressing human, α1, γ, β3 globin and bred with knockout mice for the murine α-globin and β-globin genes (10, 71, 72). This sickle cell mouse develops irreversibly sickled red cells, anemia and multiorgan
pathology similar to that found in human with sickle cells disease. However, the BERK mouse has an unbalanced chain synthesis, $\alpha/\beta$ ratio of approximately 1.25, resulting in a $\beta$-thalassemia-like condition with microcytosis (71). In addition, there is no expression of $\gamma$-globin in the BERK adult mice, leading to a more severe phenotype with more challenging breeding.

Control mice for the BERK SCD model were generated by with the same transgenic construct containing the human $\alpha$-globin, $\gamma$-globin and the human $\beta^s$-globin genes in a heterozygous mouse for the murine $\beta$-globin deletion (wild type/$\beta^s$) and they have corrected sickle cell phenotype. These are referred to as hemi-zygote mice and are often used as controls, with some notable limitations (63).

An additional SCD mouse model is the humanized transgenic “Townes” mouse. These mice were created using the same knockout-transgenic approach described above to replace the endogenous murine $\alpha$-globin and major and minor $\beta$-globin genes with a human hemoglobin transgene construct. The transgene contains the human $\alpha$-globin gene and both the human $\gamma$ and $\beta^s$-globin genes. Control mice were generated by knock-in of a construct containing the human $\gamma$-globin and the human wild type $\beta$-globin gene to obtain a mouse producing human $\alpha$-globin and $\beta$-globin (wild type) that have corrected sickle cell phenotype (84). The Townes mouse model also develops severe anemia with reduced hematocrit and high reticulocyte counts. This mouse expresses more fetal hemoglobin at birth, does not have a thalassemic phenotype and is easier to breed. Additionally, both of these transgenic models show multi-organ pathology, including pulmonary vascular congestion and alveolar thickening, liver infarction and fibrosis, glomerular congestion and afferent vessel dilation, splenic congestion and thrombosis, and microvascular congestion in the cortex of the brain (24, 27, 71, 84). Despite the severe anemia in these mice, they survive up to 15 months of age, which provides an effective model for testing therapeutic approaches for this disease.

Mouse models have been developed with special emphasis to analyze the effect of HbF expression in SCD. Patients of SCD are protected from the complications of the disease at birth and up to six months of age because the predominance of HbF during this period of time. Rational design of therapeutic strategies for $\beta$-globin disorders, such as SCD, include targets that mediate HbF induction. One of these mouse models is the NY1KO mouse. This mouse model is a knockout of both murine $\alpha$ and $\beta$ globins, and expresses exclusively human sickle hemoglobin by transgene insertion of human $\alpha$-globin,
human $\beta^s$-globin and human $\gamma$-globin. Minor differences in the $\gamma$-globin construct guides production of 3 levels of HbF (low, medium or high) (24, 71). The increase of HbF expression from less than 3% to 20% and 40% correlated with escalating improvement of hematocrit, diminution in reticulocyte counts and reduction of severity of disease and tissue damage.

Another model was developed using a transgenic $\beta^s$ mouse and adding a transgene of the $\beta$-globin sickle-Antilles ($\beta^{S\text{-Antilles}}$), a mutation originating in a family in the Caribbean. The $\beta^s$ transgene contains the human locus control region, human $\alpha_2$-globin and human $\beta^s$-globin on a mouse background of murine $\beta$-globin major deletion. The S-Antilles transgene contains the human $\beta^{S\text{-Antilles}}$ mutation which occurs at Ile$^{23}$. This bi-transgenic variant makes HbS plus HbS-Antilles which enhances the deoxygenation related polymerization of hemoglobin tetramers within the red blood cells (27, 28). Mice have a spontaneous~3.7 kilobase deletion of murine $\beta$-globin major in addition to an insertional disruption of the murine $\beta$-globin minor (28). They continue to express murine $\alpha$-globin, and it must be noted that murine $\alpha$-globin prevents polymerization of Hb tetramers nearly as effectively as $\beta$-globin. (30) These mice express approximately 60% human $\alpha$-globin, but under hypoxic conditions develop red blood cell sickle morphology, severe hemolytic anemia, and organ pathology similar to what is seen in SCD patients (27).

The array of mouse models for SCD (Table 1) recapitulate many of the features of sickle cell disease pathophysiology, including hyperhemolysis, adhesion molecule expression with vaso-occlusion, endothelial dysfunction, activation of oxidases such as xanthine oxidase and NADPH oxidases, inflammation and ischemia-reperfusion injury. (2, 14, 50-52, 66, 67, 70, 91). They provide an opportunity to invasively study the complex pathobiology of vaso-occlusion and vasculopathy and provide an avenue to probe new potential therapies.

**Pulmonary hypertension in transgenic humanized sickle cell mice**

Mouse models of SCD provide the opportunity to study the pathophysiologic mechanisms underlying the development of PH and potential therapeutic strategies. The double knockout, transgenic mouse has been used to demonstrate development of PH as a complication of chronic intravascular hemolysis. In 2006 indirect evidence of PH was shown by histological examination finding pulmonary
artery thickening in mice one to six months old who also had low hematocrit and high reticulocytosis (34).

The use of intact-chest catheterization of BERK mice ages three to five months old was able to demonstrate pulmonary arterial hypertension (PAH) with an average systolic pulmonary arterial pressure of 23 mmHg compared with 12.5 mmHg in littermate controls (44). It is important to note that this PH was correlated with increased NO consumption and eNOS uncoupling in the sickle mice (44). The Townes sickle cell mouse has been used to interrogate downstream mechanisms of development of PH. Recently PPARγ was shown to decrease as Endothelin-1 was increased in pulmonary artery endothelial cells, extracted from Townes mice, and exposed to hemin (a component of cell-free hemoglobin) (18). A tissue factor inhibition strategy was shown to decrease vascular injury in the Townes sickle mouse model, a potential target for therapy (83). Using the mouse model of sickle cell disease is a strategy to guide future therapeutic studies in PH.

**PAH therapies and application to sickle cell disease associated PH**

**Treatment of underlying sickle cell disease:**

SCD patients with PH have to adapt to both severe hemolytic anemia and increased pulmonary vascular resistance. They present with a chronically elevated cardiac output, which likely contributes to pulmonary vascular shear injury over time and increases the demands on the right ventricle in the setting of rising pulmonary vascular resistance (1). A central goal of therapy is to reduce the severity of hemolytic anemia with treatment with hydroxyurea, which increases the concentration of fetal hemoglobin, and in more severe cases or in patients who cannot tolerate hydroxyurea, through chronic red blood cell transfusions regimens. Proper transfusion therapy can improve tissue oxygen delivery and decrease the amount of sickle RBCs being synthesized. Cardiovascular complications in other hemolytic diseases have been improved with these therapeutic strategies (87). In addition, therapies that reduce HbS polymerization will reduce vaso-occlusive crises and acute chest syndrome events. It has been shown that during episodes of the acute chest syndrome the pulmonary pressures can rise and patients can develop acute right heart failure. (60) These patients suffer a high risk of death after the acute chest syndrome event, suggesting that the prevention of acute chest syndrome may reduce mortality in patients with sickle cell disease and PH. (53)
The development of therapies to treat pulmonary hypertension has evolved over the past twenty years with drugs that target the canonical pathways that regulate pulmonary vasodilation and vasoconstriction (Figures B, C).

Therapies in PAH and SCD targeting L-arginine-NO-sGC-cGMP-PDE5-I signaling nexus:

Nitric oxide is an important regulator of vascular function due to its activation of the smooth muscle cell soluble guanylate cyclase, which in turn converts GTP to cGMP. As described earlier, in sickle cell disease the NO signaling axis is disrupted at multiple steps, suggesting that therapies to enhance this pathway may improve vascular function. The supplementation of oral L-arginine has been evaluated in subjects with SCD and was shown to improve pulmonary artery systolic pressure in patients with SCD associated PAH treated for 10 days, however this has not been tested in a larger clinical trial (65). The inhalation of NO was approved by the FDA for the treatment of persistent fetal circulation in newborns, and has been shown to be effective in PAH patients (21). While inhaled NO gas has not been studied in patients with SCD and PH, a multicenter trial of inhaled NO for SCD patients admitted for veno-occlusive crisis showed no efficacy (34).

Sildenafil is a phosphodiesterase 5 inhibitor that increases the levels of the downstream amplified second messenger cGMP and promotes pulmonary vasodilation. A trial of Sildenafil for the treatment of PAH demonstrated improved exercise capacity, World Health Organization functional class, and hemodynamics in patients with symptomatic pulmonary arterial hypertension that were treated for 12 weeks (29). Machado and colleagues tested Sildenafil in a pilot study of 12 patients with SCD treated for up to 6 months. They observed improved PH and exercise capacity (56). This small pilot study lead to the multicenter, double-blind, placebo-controlled Phase 2 trial evaluating the effects of Sildenafil in subjects with SCD (55). The study’s primary endpoint was improved exercise capacity, however the study was stopped early by the data safety and monitoring board based on the observation of an increased rate of hospitalizations secondary to pain episodes compared with placebo (55). It is important to note that in the first trial using Sildenafil to treat PAH (idiopathic, congenital systemic to pulmonary shunt and associated with connective tissue disease) also had an increased incidence of myalgias, back pain and limb pain compared with placebo (29). In the long-term follow up study, the same was observed, with treatment related myalgias, back pain, limb pain, and also arthralgias noted in the
treatment group (7). After 6 months of treatment with Tadalafil (a long-acting phosphodiesterase 5 inhibitor) for erectile dysfunction there was an observed increase in myalgias, back pain and general pain compared with placebo in both the 10mg and 20mg treatment groups (9). These data suggest that there is a class effect of the phosphodiesterase 5 inhibitors on chronic back and limb pain that may have increased hospitalization rates for pain in SCD patients in the walk-PHaSST trial. Future studies will be required to ascertain whether newer agents that increase cGMP via sGC activation will have the same complications. However, these do not appear to cause myalgia or back pain in PAH or CTEPH patients (31, 32).

The potential to treat conditions in which nitric oxide levels are insufficient would have great therapeutic potential. A new class of drugs has been developed that are able to bypass nitric oxide bioavailability. These drugs are able to target soluble guanylate cyclase within smooth muscle cells directly, increasing intracellular levels of cyclic guanosine monophosphate and inducing vasodilation (Figure C). One compound stimulates the reduced form of the cyclase enzyme’s sensitivity to even low amounts of NO that is available (26). The other compound can activate the oxidized form of guanylate cyclase without NO being present. Three recent studies were able to demonstrate the effectiveness of the stimulator form for different types of pulmonary arterial hypertension. First, Riociguat for the Treatment of Idiopathic Pulmonary Arterial Hypertension was shown to be effective (32). Second, it has demonstrated improvement of chronic thromboembolic PH (CTEPH) (31). Additionally, Riociguat for patients with PH caused by systolic left ventricular dysfunction was supported by a phase IIb double-blind, randomized, placebo-controlled, dose-ranging hemodynamic study (15).

In a disease like SCD a profound amount of hemolysis exists, leading to low NO bioavailability (38), through NO scavenging by the hemoglobin-dioxygenation reaction. The oxidative stress within the vasculature renders much of the smooth muscle cell sGC oxidized and inactive. The ability of these new sGC activator medications to bypass the NO pathway and activate sGC directly and increase cGMP and inducing vasorelaxation may offer a potential therapeutic advantage for PH in hemolytic diseases. Raat and colleagues recently demonstrated that plasma free heme associated vasoconstriction, due to scavenging of NO, had little improvement using both sodium nitroprusside and Sildenafil. However, the vasoconstriction could be reversed with sGC modulators working independently of NO, and restoring cGMP mediated vasodilation in rats (75).
**Endothelin1 pathway:**

The inflammatory cytokine Endothelin-1 (ET-1) induces vasoconstriction, inflammation, fibrosis and cellular proliferation when it binds to its receptors ET-A and ET-B (62). Levels of ET-1 are elevated in patients with SCD suffering from vaso-occlusive crisis and PH (42). In erythrocytes when ET-1 binds to ET-B it activates the membrane Gardos channels, which causes dehydration of the RBCs and increases hemolysis (80, 81). A recent small retrospective case series showed safety and efficacy of ET receptor blockers in SCD associated PH, with significant improvement in 6MWD and a trend towards significant decrease in NT-BNP and LDH indicating its usefulness in this population (62). A placebo controlled clinical trial of Bosentan was stopped early secondary to withdrawal of support by the sponsor, but did report safety of the drug in this patient population. (10)

Epoprostenol is an extremely short acting, but potent, pulmonary vasodilator. This therapy was proven effective with 12 weeks of continuous infusion in patients with primary PH, with improved 6MWD, mPAP and PVR (11). The use of this intravenous prostacyclin has been shown to acutely decrease PVR in SCD patients with PH diagnosed during RHC (20). Further development of the prostanoids has produced subcutaneous, inhaled and oral formulations that have been effective in improving symptoms and exercise performance in clinical trials (30). There is no data with SCD patients with associated PH using these agents.

**New medications in development:**

ACT-293987, otherwise known as Selexipag, is a non-prostanoid selective IP agonist, and has potential for potent vasodilation without as many side effects as prostacyclins, due to its target selectivity. It is delivered in an oral form, which has an easier delivery than many other prostanoids. This drug has only been tested in primary PAH, and not in SCD patients. In a Phase II trial in patients with PAH, there was a significant decrease in PVR as compared with patients treated with placebo (86).

**Conclusions**

Patients with SCD in the developed world are now living long into adulthood. This change in the survival of patients with this disease results in the presentation of new long-term vasculopathic
complications such as systemic hypertension and PH, which are now recognized as major risk factors for
death in adults with SCD. A major risk factor for development of chronic vasculopathy and PH in patients
with SCD is hemolytic anemia (37), caused by the pathological effects of plasma cell free hemoglobin and
other eDAMPs. Screening patients with SCD for PH for early diagnosis and disease modifying therapy is
recommended.
REFERENCES:


Figure A
Panel 1: Normal vascular conditions. Red blood cells (RBC) circulate avoiding the cell free zone by laminar flow. Platelets accompany RBC inactive from nitric oxide (NO) inhibition. Endothelial NO synthase (eNOS) produces NO from substrate L-Arginine (L-Arg), making L-Citruline (L-Cit) and NO. NO has a paracrine effect traveling into smooth muscle cells (SMC) which then activate soluble guanylate cyclase (sGC) forming cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) leading to vasorelaxation and increasing blood flow distally. Panel 2: Sickled cells undergo intravascular hemolysis releasing Arginase, ADP and hemoglobin tetramers into vascular space. Cell Free Hemoglobin (CFH) enters cell free zone scavenging NO and forming reactive oxygen species (ROS). Arginase consumes substrate L-Arg. ADP activates platelets via P2Y receptors. Panel 3: Hypoxia/re-oxygenation/hemolysis activates oxidase activity. Ischemia/reperfusion activates endothelial xanthine oxidase (XO) in addition to mobilizing soluble hepatic XO which binds to vascular endothelial cells and forms ROS from excess purine nucleotides from increased RBC formation/enucleation. Leukocyte and endothelial derived NADPH oxidase (NOS) forms ROS from unbound oxygen. ROS inhibit sGC via oxidation, preferential superoxide (O2-) formation by eNOS uncoupling and O2- scavenging of NO within the cell free zone to form peroxynitrite (OONO-). Panel 4: Intravascular hemolysis activates the inflammasome. Uric Acid formed by XO binds to nucleotide-binding oligomerization domain–like receptor (NOD-LR) which promotes release of IL1-β. Cell free heme (CFH) activation of TLR-4 receptors. Both mechanisms lead to sterile inflammation. ROS and CFH release by cell free hemoglobin activate polymorphonuclear cells (PMN) which release DNA nets. PMNs also release placental growth factor (PGF) which induces cellular proliferation. Products CFH, uric acid and ATP are considered danger associated molecular pattern molecules (DAMPs) when associated with intravascular hemolysis.

Figure B
Vasoconstriction and Vasodilation pathways and therapies for pulmonary hypertension (PH). Cyclic guanosine monophosphate (cGMP) leads to vasodilation and inhibits cellular proliferation in smooth muscle cells via activation of soluble guanylate cyclase (sGC) by nitric oxide (NO). Inhaled NO, oral Nitrite and Nitrate, oral L-arginine or tetrahydrobiopterin (BH4), and phosphodiesterase 5 (PDE5) inhibitors (sildenafil/tadalafil) treat PH through the NO-sGC-cGMP pathway. Prostacyclins, produced from Arachidonic Acid by Cyclooxygenase (COX) in endothelial cells, target the prostanoid receptor (IP) on smooth muscle cells leading to formation of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) by adenylyl cyclase. cAMP promotes vasodilation and inhibits cellular proliferation in SMC. Epoprostenol is a prostanoid derivative and Selexipag is an IP receptor agonist that can treat PH through the prostacyclin pathway. Endothelin-1 (ET-1) promotes vasoconstriction and vascular remodeling through binding to ET-A and B receptors of the SMC. Ambrisantan binds to ET-A and Bosentan binds to both ET-A and B. Both ET-1 blockers have been used to treat PH in the Endothelin pathway.

Figure C:
New therapies that target the NO signaling pathway. Soluble guanylate cyclase (sGC) with in smooth muscle cells (SMC) can be stimulated by NO which binds to the heme moiety within the heterodimer, activating its cyclase function, producing cyclic guanosine monophosphate (cGMP) and promoting vasodilation. sGC can be oxidized by reactive oxygen species (ROS) such as superoxide (O2-). The oxidized form does not respond to NO binding and is quickly degraded by proteasomes within the cell. sGC activator targets the oxidized (heme-free) form of sGC and salvages its degradation while activating the cyclase and inducing production of cGMP promoting vasodilation.

Table 1
Transgenic murine models of sickle cell anemia.
Figure A
Panel 1: Normal vascular conditions. Red blood cells (RBC) circulate avoiding the cell free zone under laminar flow. Platelets accompany RBC are inhibited by endothelial derived nitric oxide (NO). Endothelial NO synthase (eNOS) produces NO from the substrate L-Arginine (L-Arg), making L-Citruline (L-Cit) and NO. NO has a paracrine effect diffusing into smooth muscle cells (SMC) which then activates soluble guanylate cyclase (sGC) forming cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP), leading to vasodilation. Panel 2: Sickled cells undergo intravascular hemolysis releasing Arginase, ADP and hemoglobin tetramers into the vascular space. Cell Free Hemoglobin (CFH) enters the cell free zone scavenging NO and forming reactive oxygen species (ROS). Arginase consumes substrate L-Arg. ADP activates platelets via P2Y receptors. Panel 3: Hypoxia/re-oxygenation/hemolysis activates oxidase activity. Ischemia/reperfusion activates endothelial xanthine oxidase (XO) in addition to mobilizing soluble hepatic XO which binds to vascular endothelial cells and forms ROS from excess purine nucleotides from increased RBC formation/enucleation. Leukocyte and endothelial derived NADPH oxidase (NOS) forms ROS. ROS inhibits sGC via oxidation. Additional superoxide (O$_2^-$) formation by eNOS uncoupling and O$_2^-$ scavenging of NO within the cell free zone forms peroxynitrite (OONO-). Panel 4: Intravascular hemolysis activates the inflammasome. Uric Acid formed by XO binds to nucleotide-binding oligomerization domain–like receptor (NOD-LR) which promotes release of IL1-β. Cell free heme (CFH) activates the TLR-4 receptors. Both mechanisms lead to sterile inflammation. ROS and CFH release by cell free hemoglobin activate polymorphonuclear cells (PMN) which release DNA nets. PMNs also release placental growth factor (PGF) which induces cellular proliferation. Products CFH, uric acid and ATP are considered danger associated molecular pattern molecules (DAMPS) when associated with intravascular hemolysis.
**Figure B**

Vasoconstriction and Vasodilation pathways and therapies for pulmonary hypertension (PH). Activation of soluble guanylate cyclase (sGC) by nitric oxide (NO) increases cyclic guanosine monophosphate (cGMP) levels and leads to vasodilation and inhibition of cellular proliferation in smooth muscle cells. Inhaled NO, oral Nitrite and Nitrate, oral L-arginine or tetrahydrobiopterin (BH4), and phosphodiesterase 5 (PDE5) inhibitors (sildenafil/tadalafil) modulate PH through the NO-sGC-cGMP pathway. Prostacyclins, produced from Arachidonic Acid by Cyclooxygenase (COX) in endothelial cells, target the prostanoid receptor (IP) on smooth muscle cells leading to the activation of adenylate cyclase (AC), which forms cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). cAMP promotes vasodilation and inhibits cellular proliferation in SMC. Epoprostenol is a prostanoid derivative and Selexipag is a new IP receptor agonist that can treat PH through the prostacyclin pathway, activating AC. Endothelin-1 (ET-1) promotes vasoconstriction and vascular remodeling through binding to ET-A and B receptors of the SMC. Ambrisantan binds to ET-A and Bosentan binds to both ET-A and B. Both ET-1 blockers have been used to treat PH in the Endothelin pathway.

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