

Intravascular hemolysis and the pathophysiology of sickle cell disease

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Hemolysis is a fundamental feature of sickle cell anemia that contributes to its pathophysiology and phenotypic variability. Decompartmentalized hemoglobin, arginase 1, asymmetric dimethylarginine, and adenine nucleotides are all products of hemolysis that promote vasomotor dysfunction, proliferative vasculopathy, and a multitude of clinical complications of pulmonary and systemic vasculopathy, including pulmonary hypertension, leg ulcers, priapism, chronic kidney disease, and large-artery ischemic stroke. Nitric oxide (NO) is inactivated by cell-free hemoglobin in a dioxygenation reaction that also oxidizes hemoglobin to methemoglobin, a non-oxygen-binding form of hemoglobin that readily loses heme. Circulating hemoglobin and heme represent erythrocytic danger-associated molecular pattern (eDAMP) molecules, which activate the innate immune system and endothelium to an inflammatory, proadhesive state that promotes sickle vaso-occlusion and acute lung injury in murine models of sickle cell disease. Intravascular hemolysis can impair NO bioavailability and cause oxidative stress, altering redox balance and amplifying physiological processes that govern blood flow, hemostasis, inflammation, and angiogenesis. These pathological responses promote regional vasoconstriction and subsequent blood vessel remodeling. Thus, intravascular hemolysis represents an intrinsic mechanism for human vascular disease that manifests clinical complications in sickle cell disease and other chronic hereditary or acquired hemolytic anemias.

Introduction

Patients with sickle hemoglobinopathies have variable phenotypes, with different pain frequencies and severity and pleiotropic complications, including lung injury, stroke, cutaneous leg ulceration, kidney injury with proteinuria, osteonecrosis, and systemic and pulmonary hypertension (PH). These phenotypes result from erythrocyte injury caused by sickle hemoglobin (HbS) and its deoxygenation-induced polymerization. Erythrocyte injury leads to extra- and intravascular hemolysis, endothelial dysfunction and vasculopathy, and occlusion of small and large blood vessels, producing tissue ischemia/reperfusion injury and inflammation. Damage to circulating erythrocytes occurs with wide diversity amongst individuals (1). This heterogeneity arises from differences in intrinsic characteristics of sickle erythrocytes, like heterocellular fetal hemoglobin (HbF) distribution, HbS concentration (2), hydration, and density (3, 4), and the cell's environmental transitions from macro- to microcirculation, laminar to turbulent

flow, normoxia to hypoxia, isotonic to hypertonic environment, and acidotic to alkalotic milieu. Multiple components contribute to sickle hemoglobinopathy pathophysiology, including primary components arising from HbS polymerization and secondary components that are downstream effects of the HbS polymer. Understanding how these components' complexity is compounded by genetic and environmental modulation provides insight into the well-known clinical heterogeneity of sickle cell disease (SCD).

A cardinal feature of SCD pathogenesis involves inflammation, accompanied by heterocellular leukocyte-platelet-erythrocyte-endothelial adhesive events that trigger vaso-occlusive episodes, acute organ ischemia, and reperfusion injury. Twenty-five years ago, epidemiological studies identified leukocytosis, lower HbF levels, and higher total hemoglobin levels as risk factors associated with increasing incidence of acute painful episodes and acute chest syndrome (ACS) (5). The independent association of high total hemoglobin levels with more pain, ACS events, and osteonecrosis was never mechanistically explained; however, it was implied to be a result of increased blood viscosity (Table 1). Recent epidemiological studies found that lower hemoglobin levels and higher intensity of steady-state hemolytic anemia consistently associate with vasculopathic complications of disease, such as stroke, leg ulcers, PH, priapism, and renal failure. This suggests that certain subphenotypes of SCD relate more to hemolytic anemia severity rather than sickle vaso-occlusion. The reader is referred to recent reviews describing the exceptional strides made in understanding the roles of red cell rigidity (6), inflammation, and cell adhesion in sickle vaso-occlusion (7–9). Here, we review the complementary role of intravascular hemolysis and anemia.

Conflict of interest: M.H. Steinberg has equity ownership of more than \$10,000 in publicly held companies in the health care field and has provided testimony as an expert witness in medical-legal cases. G.J. Kato is listed as a coinventor on a patent application by the NIH for the formulation of topical sodium nitrite (PCT/US2015/060015). M.T. Gladwin is listed as a coinventor on an NIH government patent for the use of nitrite salts in cardiovascular diseases (US 8,927,030 B2). M.T. Gladwin receives research support from Aires/Mast Therapeutics on the development of a phase II proof-of-concept trial using inhaled nitrite for pulmonary arterial hypertension and leads a collaborative research and development alliance with Bayer Pharmaceuticals. G.J. Kato and M.T. Gladwin receive research support from Bayer Pharmaceuticals.

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Table 1. Subphenotypes of SCD and their association with hyperhemolysis, α -thalassemia, and HbF

Clinical features	Effect of hyperhemolytic subphenotype	Effects of α -thalassemia	Protection by HbF
Painful episodes/dactylitis	Reduces risk (42, 151)	Increases risk (151, 152)	Protective (5, 32)
Acute chest syndrome	Neutral (42)	Increases risk (152)	Protective (31, 32)
Leg ulcers	Increases risk (42, 153)	Reduces risk (21)	Equivocal (21, 153, 154)
Osteonecrosis	Reduces risk (42)	Increases risk (155, 156)	Equivocal (22, 157–161)
Priapism	Increases risk (42)	Reduces risk (71)	Not protective (71, 162)
Renal function/albuminuria/hemoglobinuria	Increases risk (24, 45)	Reduces risk (24, 43, 165, 164)	Not protective (164–129)
Stroke, increased TCD velocity	Increases risk (23, 42, 170)	Reduces risk (23, 170–173)	Not protective in children; possibly protective in adults (174–177)
Bilirubinemia/cholelithiasis	Increases risk (178, 179)	Reduces risk (180, 181)	Protective (159, 182)
Retinopathy	Neutral (18)	Equivocal (183, 184)	Possibly protective (185)
Sickle vasculopathy/TR velocity/systemic hypertension	Increases risk (19, 42, 49, 64)	Equivocal (186)	Not protective (19, 49, 62, 68, 187)
Mortality	Increases risk (19, 39, 42, 47, 49, 64, 188)	Protective (189)	Protective (190)

Hyperhemolysis is inferred from a combination of increased serum concentration of indirect bilirubin and lactate dehydrogenase. α -Thalassemia was ascertained by gene analysis. For nearly every subphenotype it is possible to find some contradictory evidence because of differences in cohort age distributions, sample size, phenotype definitions, and analytical approaches. Because of space limitations, many studies are not included. For most subphenotypes, both children and adults are included. TCD, transcranial Doppler; TR, tricuspid regurgitant.

Unless specified, in this Review “hemolysis” and “intravascular hemolysis” are used interchangeably.

The hemolysis hypothesis

Nine years have passed since we proposed that intravascular destruction of sickle erythrocytes is pathogenetically related to certain common complications of SCD, igniting a long-smoldering debate on the mechanistic basis of these associations (10–12). The crux of the hypothesis was a general appreciation that products of intravascular hemolysis damage the vascular system (13). More specifically, it proposed that nitric oxide (NO) depletion in the microcirculation resulted from intravascular hemolysis–driven release of cell-free hemoglobin into the plasma that reacted with NO via the well-known dioxygenation reaction to form inert nitrate. This reaction occurs in vitro (14) and is promoted by blood substitutes in vivo (15), and its occurrence in SCD is supported by in vitro and in vivo evidence, summarized later in this Review (16). NO is a free radical produced enzymatically by a family of NO synthases (NOSs) during the conversion of arginine to citrulline. Endothelial NO, produced by endothelial NOS3, diffuses to adjacent smooth muscle, where it binds and activates the heme of soluble guanylate cyclase, which subsequently converts GTP to cGMP. This activation of cGMP-dependent protein kinases produces vasodilation by causing calcium sequestration and perivascular smooth muscle relaxation. NO is also depleted during intravascular hemolysis when arginase is liberated from erythrocytes, destroying arginine, the substrate for NOS (17), and by reactions of NO with ROS that are generated during intravascular hemolysis. Compounding the effects of these NO- and arginine-scavenging pathways, lysed red cells release asymmetric dimethylarginine, an endogenous inhibitor of NOS (18). A role for intravascular hemolysis in promoting endothelial dysfunction was bolstered by epidemiological cohort studies linking laboratory biomarkers of the intensity of hemolytic anemia and risk of developing specific complications of SCD, including PH (19), cutaneous leg ulceration (20, 21), priapism (22), stroke (23), and, recently, pro-

teinuria and renal insufficiency (24–26). In contrast, as mentioned earlier, other complications were associated with lower hemolysis rates and higher steady-state hemoglobin levels, including the rate of vaso-occlusive painful episodes, ACS, and osteonecrosis (Table 1). Unlike hemolysis, traditional established risk factors for vaso-occlusive episodes, such as steady-state leukocytosis (27, 28) and high hemoglobin levels (29), do not accurately predict the above-mentioned vasculopathic events and mortality observed as the patient population ages. To date, no alternative mechanism has been proposed to explain the divergent associations between the severity of hemolytic anemia and specific clinical complications.

With a decade of new data to review, we now reappraise the relationship between intravascular hemolysis and the pathophysiology of SCD and further extend the role of intravascular hemolysis and NO scavenging to other diseases.

Evidence that hemolysis modulates SCD subphenotypes

SCD phenotypes are expressed in common and rare subphenotypes. Some subphenotypes are attributed to sickle vaso-occlusive events triggered by adherent sickle erythrocytes and are closely related to packed cell volume, blood viscosity, and inflammation/intracellular adhesion. Other events are a presumed consequence of intravascular hemolysis of injured sickle cells. Table 1 lists common subphenotypes of disease and their epidemiological associations with biomarkers of hemolytic anemia and inflammation/viscosity/vaso-occlusion. HbF and α -thalassemia are the two principal modulators of the SCD phenotype. HbF has its most robust effects on subphenotypes associated with sickle vaso-occlusion, including ACS (30–32). Failure of HbF to afford similar levels of protection for hemolysis-associated subphenotypes might be a consequence of insufficient HbF in some cells, which allows continued intravascular hemolysis and endothelial injury over long exposures (33, 34).

α -Thalassemia modulates the phenotype of SCD by reducing hemolysis (35). α -Thalassemia reduces mean cell hemoglobin

concentration and erythrocyte density, thereby reducing the tendency of deoxy-HbS to polymerize (35). In compound heterozygotes for α -thalassemia and sickle cell anemia (SCA, characterized by homozygosity for the HbS gene), hemoglobin levels are higher and the prevalence of subphenotypes associated with hemolytic anemia are reduced in comparison with SCA alone; in contrast to SCA, the prevalence of vaso-occlusive pain crisis and the prevalence of ACS are increased (36). Consistent with this observation, in a placebo-controlled clinical trial in adults with SCA, a Gardos channel inhibitor significantly reduced hemolysis, increased hemoglobin levels, and increased the rate of vaso-occlusive painful episodes (37). Reduction of hemolysis by the Gardos channel inhibitor was associated with significant decline in serum N-terminal pro-B-type natriuretic peptide (NT-proBNP) (38), potentially consistent with decreased pulmonary artery pressure, but also possibly caused simply by improved anemia and lower cardiac preload (39). The results of this antihemolytic pharmacological intervention substantially phenocopy SCA- α -thalassemia, further supporting phenotypic modulation of SCD by hemolysis and anemia. Other than increased blood viscosity, only increased adhesiveness is hypothesized to mediate the increased risk of vaso-occlusion in SCA- α -thalassemia (40), but no published evidence supports this alternative hypothesis.

Many studies have confirmed the association of indirect markers of hemolysis with certain complications of disease. Hemolytic rate in SCD has been measured directly by labeled-erythrocyte survival studies (34, 41). More often, indirect surrogates of red cell lifespan, including plasma hemoglobin, total hemoglobin, erythrocyte microparticles, serum bilirubin, reticulocyte count, lactate dehydrogenase (LDH), alanine aminotransferase (AST), and urine hemosiderin or cell-free hemoglobin, have been used singly and in combination to estimate the hemolysis intensity (20, 25). Composite indices characterizing hemolysis severity were validated by comparison with the plasma levels of cell-free hemoglobin and red blood cell microparticles. These measures correlate appropriately with hemoglobin and reticulocyte counts and are decreased in patients with high HbF or with α -thalassemia (25). Based on plasma hemoglobin measurements, the fraction of intravascular hemolysis ranges from less than 10% to more than 30%, while total plasma hemoglobin concentrations (reported here in terms of heme concentration) range from 0.25 to more than 20 μ M (16). As discussed later, these concentrations are sufficient to scavenge NO and perhaps trigger sickle vasculopathy.

Hyperhemolysis-associated SCD subphenotypes

A suggested paradigm proposes a gradient of hemolysis among SCD patients, with the highest quartile of this gradient sometimes referred to as hyperhemolysis (19–23, 25, 26, 41, 42–47). This gradient results from the differential distribution of HbF concentrations among sickle erythrocytes, the presence or absence of α -thalassemia mutations, and perhaps other genetic determinants that directly or indirectly affect erythrocyte lifespan (36). Included in the vasculopathic subphenotypes associated with hyperhemolysis are PH, cerebrovascular disease, leg ulcers, priapism, and sickle nephropathy (20). Tricuspid regurgitant velocity (TRV) and serum NT-proBNP are markers of pulmonary vascular disease and myocardial wall stress and are consistently associated with

hyperhemolysis (19, 39, 44, 48–56). PH, either screened for with TRV or definitively ascertained by right heart catheterization, is closely associated with the intensity of hemolysis and mortality (discussed below). Some aspects of the hemolytic phenotype, like abnormally high TRV and PH confirmed by right heart catheterization, are seen in other hemolytic anemias where intravascular hemolysis is common (and vaso-occlusive crisis is absent) like β -thalassemia (57). Similarly to SCD (58), these other hemolytic disorders also feature a hypercoagulable state with evidence of microthrombotic disease in the pulmonary vasculature (59).

Hemolytic anemia and vasculopathic complications

Numerous cohort studies evaluated and confirmed the association of hemolytic anemia severity with increasing pulmonary pressures estimated by TRV and directly measured by right heart catheterization. Many of these studies also evaluated the relationship between estimated or directly measured pulmonary artery pressures and reduced exercise capacity and/or risk of death. These studies include the NIH-PH (19), Duke (60), UNC (49), MSH (39), CSSCD (54), PUSH (50, 61), and Walk-PHASST (51) cohorts, a Greek cohort (62), and a recent 656-SCD-patient echocardiographic screening study in Créteil, France (63). The analysis of more than 600 screening patients in both the Walk-PHASST and Créteil cohorts found similar associations between indices of hemolytic anemia, high TRV, and risk of death (51, 63). These associations were largely confirmed in right heart catheterization studies (64–66).

Severity of hemolytic anemia was associated not only with risk of precapillary PH, but also with risk of postcapillary PH. The latter was observed with echocardiographic markers of heart failure with preserved ejection fraction (67–70) and right heart catheterization (64–66). The involvement of left ventricular disease complicates the diagnosis, clinical management, and prognosis in SCD, as discussed below.

While hemolytic anemia is an independent risk factor for vasculopathic complications, hemolysis does not occur in isolation. Priapism and leg ulcers occur more frequently in SCD patients than in patients with other hemolytic diseases like paroxysmal nocturnal hemoglobinuria (PNH), spherocytosis, β -thalassemia, and pyruvate kinase deficiency, which surely represents the contribution of unique characteristics of the sickle erythrocyte, sickle vaso-occlusion, and inflammatory damage to intravascular hemolysis-provoked injury. This is particularly evident in the epidemiology of priapism, in which indices of both hemolytic anemia and inflammation are associated with this clinical manifestation (71). A nexus between hemolysis and sickle vaso-occlusion might lay in the increased adhesivity of the sickle reticulocyte, sickle erythrocyte lysis in vaso-occluded regions (discussed below), and downstream inflammatory effects of intravascular hemolysis products, like heme, that drive sterile inflammation.

Elevated TRV and SCD complications

TRV can be quantified by Doppler echocardiography and used to estimate pulmonary artery systolic pressure. This value is a predictive physiological biomarker and a widely used screening test for PH. While TRV has important limitations in sensitivity and specificity, it is a continuous variable that is inversely proportional to exer-

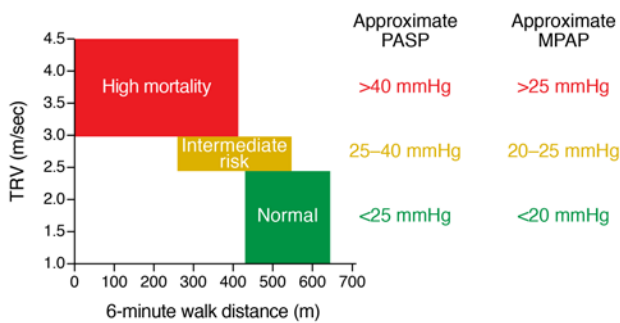


Figure 1. Tricuspid regurgitant velocity on echocardiogram is a physiological biomarker that predicts survival and functional outcomes. Tricuspid regurgitant velocity (TRV) that is less than two SD below the population mean is in the normal range (<2.5 m/s), is associated with normal calculated pulmonary artery systolic pressures (PASP, <25 mmHg), and generally corresponds to a mean pulmonary artery pressure (MPAP, <20 mmHg), good long-term survival, and good exercise tolerance, as indicated by a higher 6-minute walk distance (>400 m). Conversely, highly elevated TRV that is more than 3 SD above the mean (≥ 3 m/s) is strongly associated with poor exercise tolerance with lower 6-minute walk distance (<400 m), pulmonary artery systolic pressure greater than 40 mmHg, mean pulmonary artery pressure greater than 25 mmHg on right heart catheterization, and significantly poorer long-term survival. Intermediate TRV level (2.5–2.9 m/s) is associated with intermediate risk of exercise intolerance and mortality. Figure is adapted with permission from the *Journal of the American Medical Association* (39) and the *American Journal of Respiratory and Critical Care Medicine* (74).

cise capacity measured by the 6-minute walk distance and directly related to rising risk of death in adults with SCD (19, 49, 51, 60, 63, 72). However, clinical decision making demands widely used categorical classifications. TRV = 2.5 m/s represents 2 SD above the population mean; TRV ≥ 3 m/s is 3 SD above the mean. SCD patients with TRV ≥ 2.5 m/s have a lower exercise capacity and survival time (Figure 1). For example, in the Créteil study, probability of death was low in SCD patients with a TRV ≤ 2.5 m/s, while a TRV ≥ 2.5 m/s was associated with a hazard ratio of 6.81 in multivariate analysis and rose linearly above this value with a 50% probability of death at TRV = 3.2 m/s (63). These findings are consistent with other large cohort studies (19, 51, 63, 72), as results from both the NIH-PH cohort and the Paris cohort suggest that approximately 64%–75% of SCD patients with TRV ≥ 3 m/s have PH defined by pulmonary artery catheterization documentation of mean pulmonary artery pressure greater than 25 mmHg, approximately 3 SD above the normal mean value (64, 65). SCD patients who fall into the intermediate category of TRV = 2.5–2.9 m/s (between 2 and 3 SD from the mean) have intermediate exercise capacity and mortality, falling squarely between the TRV ≤ 2.5 m/s group and the TRV ≥ 3 m/s group. Catheterization studies indicated that about 25% of these patients have a mean pulmonary artery pressure greater than 25 mmHg, supporting the low but significant predictive value of an intermediate TRV for identifying patients at risk of PH (65). Thus, the TRV category is predictive of the risk of both PH and mortality. TRV ≥ 2.5 m/s also predicts a higher risk of leg ulcers (46, 60), thrombosis (58), proteinuria (60, 73), and exercise intolerance (74, 75), suggesting that it also serves as a marker for more general vasculopathy.

Meta-analysis helped sort out the many different studies of the prevalence and risk of high TRV in SCD. This meta-analysis included 45 studies from 15 countries and 6,109 patients (47).

Prevalence of elevated TRV ≥ 2.5 m/s was 21% in children and 30% in adults. In random-effects meta-analyses, the 6-minute walk was 30.4 m less in patients with elevated TRV than in those without elevated TRV, and the associated mortality hazard ratio was 4.9. Mortality among high-TRV SCD patients seems to be limited to adults, suggesting the testable hypothesis that high TRV in childhood predicts future risk of mortality later in adulthood. Mortality estimates are likely becoming more accurate as the number of screened adults and duration of follow-up increase.

PH and mortality in SCD

In general, right heart catheterization is required for a definitive diagnosis of PH, which is defined by a mean pulmonary artery pressure of at least 25 mmHg, although it is increasingly appreciated that mean pressures between 20 and 25 mmHg are not normal and might predict reduced exercise capacity and risk. Three SCD hemodynamic studies, from the US, Brazil, and France, evaluated the prevalence of PH defined by right heart catheterization in SCD and examined associated clinical risk factors and prospective risk of death. In the study with the longest follow-up times, performed at the NIH, 531 SCD patients were evaluated with a median follow-up time of 4.7 years and a maximum of 11 years for surviving subjects (64, 76). In this cohort, 84 right heart catheterizations were performed. Fifty-five of 531 SCD subjects (10.4%), or 55 of 84 (65.5%) of those who underwent catheterization, had PH; slightly more than half had pulmonary arterial hypertension (precapillary), and the other half had pulmonary venous hypertension. PH was associated with higher LDH levels and lower hemoglobin levels, higher prevalence of leg ulcers and renal insufficiency, and lower exercise capacity, defined by worse functional classification and lower 6-minute walk distance (64, 76).

Survival estimates for SCD patients with PH was 63% at 5 years, compared with 83% for SCD patients with normal right heart catheterization. Death certificates were available for 65% of SCD patients who died, and 80% of these reported right heart failure or sudden cardiac death, a cause of death seen commonly in the general pulmonary arterial hypertension population (77). Multivariate analysis of hemodynamic variables identified pulmonary vascular resistance, transpulmonary gradient, and pulmonary artery systolic and diastolic pressures as predictors of mortality. SCD patients with PH who died had worse hemodynamic values than survivors: mean pulmonary artery pressures of 39 ± 9 versus 33 ± 7 mmHg ($P < 0.001$), transpulmonary gradient of 25 ± 10 versus 17 ± 8 mmHg ($P = 0.003$), and pulmonary vascular resistance of 279 ± 164 versus 189 ± 127 dyn·s/cm⁵ ($P = 0.017$) strongly suggested that mortality rate in adults with SCD is proportional to severity of precapillary pulmonary vascular disease (64, 76).

In the Brazilian study, 80 SCD patients were screened, and 26 with elevated estimated pulmonary artery systolic pressures by Doppler echocardiography had right heart catheterizations (66). Ten percent of patients had PH, with worse survival compared with the remaining patients. Patients with PH had lower hemoglobin and higher LDH levels, proteinuria, renal insufficiency, and lower 6-minute walk distance. The third study, in France, was also consistent with primary observations from the previously discussed trials (65). After exclusion of adults with greater disease severity, 6% of all screened adults had PH (46% precapillary and

Table 2. A comparison of SCD cohorts studied with right heart catheterization

Population characteristics	NIH-PH (64)	Paris (65)	São Paulo (66)
Exclusions	None	Lung, liver, renal disease	None
Prevalence of TRV \geq 2.5 m/s	32%	27%	40%
Prevalence of MPAP \geq 25 mmHg	11%	6%	10%
Findings in SCD PH vs. others			
Age	Older	Older	Older
History of leg ulcers	Yes	Yes	Yes (significant only with TRV)
History of frequent VOC or ACS	No	No	No
NT-proBNP	High	High	-
Hemoglobin	Low	Low	Low
LDH	High	High	High
AST	High	High	High
Alkaline phosphatase	High	High	-
Direct bilirubin	High	High	-
Creatinine	High	High	High
6MWD	Low	Low	Low
NYHA functional class	High	High	-
Mortality	High	High	High

VOC, vaso-occlusive crisis; 6MWD, 6-minute walk distance; NYHA, New York Heart Association.

54% postcapillary). Exclusion of subjects likely underestimated the true prevalence of PH, because markers of renal insufficiency, hepatic dysfunction, and iron overload are independent risk factors for high estimated pulmonary artery pressure in SCD. Patients with right heart catheterization–documented PH had lower hemoglobin and higher LDH values and higher prevalence of prior leg ulcers, and all deaths occurred in this subgroup (65).

All of these right heart catheterization screening studies likely underestimate PH prevalence, as not all patients with elevated TRV agreed to catheterization. The differences and similarities between these three studies are summarized in Table 2.

Evidence linking cell-free hemoglobin to SCD complications

Epidemiological studies of the associations between PH and other vasculopathic subphenotypes of SCD have been supported by mechanistically focused studies. The primary hypothesis that intravascular hemolysis releases cell-free plasma hemoglobin and arginase 1 to inactivate the NO signaling axis and redox balance was studied over the last 10 years in animal models and in vivo in humans. In addition to studies in humanized transgenic sickle cell mice, studies have now been performed in other diseases with significant intravascular hemolysis, including murine models of PNH, thalassemia, and malaria, and aged-blood transfusion models. Many of the studies test causality, with the addition of hemoglobin and hemoglobin inhibition by oxidation and haptoglobin therapy. We briefly review the preclinical and clinical experimental evidence below.

Vasomotor defect in SCD. SCD patients and sickle transgenic mice have impaired vasodilator responses to NO that are proportional to levels of plasma hemoglobin (16, 78–80). In humans, impaired NO signaling, directly measured via venous occlusion strain-gauge plethysmography, correlates with high levels of plasma hemoglobin and LDH (16). This was recently confirmed in a study of SCD patients correlating high plasma hemoglobin levels

with TRV elevations and impairments in flow-mediated vasodilation, a measure of endothelial NOS–dependent vasodilation (80). In the Berkeley sickle mouse, high plasma hemoglobin levels correlated with impaired blood flow responses to infusions of the NO donor sodium nitroprusside (81). In a recent study, a specific hemoglobin-scavenging peptide that depleted the levels of plasma hemoglobin restored endothelial NOS–dependent vasodilation (82).

PH in other animal models of hemolysis. Multiple mouse models of hemolysis exhibited increased plasma hemoglobin and increased plasma NO scavenging and developed spontaneous PH and right heart failure, including Berkeley sickle cell (78), spherocytosis (83), α -thalassemia (84), PNH (85), and alloimmune hemolysis mice (78).

NO resistance. NO resistance is characterized by impaired vasodilatory responses to infusions of NO donors, pulmonary and systemic vasoconstriction, and PH. In animals, inducing intravascular hemolysis or infusing hemoglobin or hemolysate can produce experimental NO resistance (86–89). This effect is attributable to hemoglobin and NO scavenging, as it can be blocked by oxidation of hemoglobin to methemoglobin with inhaled NO gas or by binding and clearance of hemoglobin with haptoglobin (86, 89–91). Acute intravascular hemolysis induced by hypotonic water infusion in mice inhibits NO signaling and causes inflammation, an effect phenocopied by acute NO inhibition using chemical NO scavengers and reversed by an NO donor and haptoglobin (92). Haptoglobin prevents extravasation of free hemoglobin into interstitial spaces, where it scavenges NO (91).

Stored blood and vasomotor defect. Infusing aged stored blood into rodents (89, 93, 94) and humans (90, 95) causes intravascular hemolysis, pulmonary and systemic hypertension, and vascular endothelial dysfunction (due to impaired NO signaling) that can be blocked by haptoglobin (96). These effects are not always observed in models or patient populations in which hemolysis is not as severe, the dose of transfused red cells is not large, and the existing compensatory reserves of haptoglobin, hemopexin, and catalytic antioxidants are not as chronically depleted as in SCD.

Malaria endothelial dysfunction. Animal and human malaria is associated with intravascular hemolysis, increases in plasma hemoglobin, and impaired NO signaling. Higher level of plasma hemoglobin correlates in patients with impaired endothelial function (97, 98) and increased estimated pulmonary artery pressures (99). In animal models, it is linked to lowered NO scavenging and increased risk of death (21). Inhaled NO is protective in rodent models of malaria (21, 100, 101).

Hemolysis, plasma heme, and erythrocyte danger-associated molecular patterns

Free heme, another product of intravascular hemolysis, is released from free hemoglobin upon oxidation. Free heme is increasingly

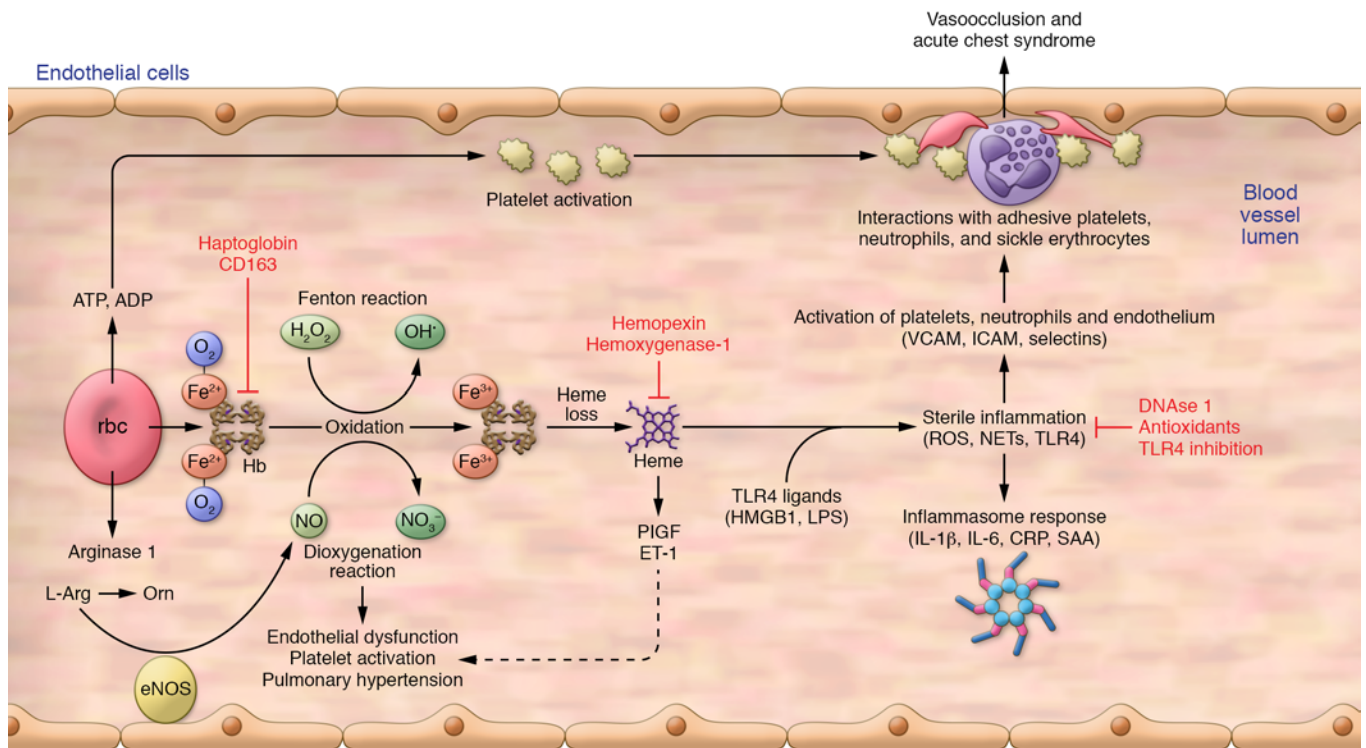


Figure 2. Contribution of intravascular hemolysis to vasculopathy and vasoocclusion. Intravascular hemolysis produces free hemoglobin, which drives Fenton reactions to produce oxidants and scavenges NO by a dioxygenation reaction. Intravascular hemolysis also releases red cell arginase 1 into plasma, where it can deplete plasma L-arginine (L-Arg), the required substrate for NO production by eNOS. Oxidized hemoglobin releases free heme, which can activate release of placenta growth factor (PIGF) and endothelin-1 (ET-1). These combined pathways contribute to chronic vasculopathy, platelet activation, and pulmonary hypertension. Heme also primes the innate immune system to acute rises in endogenous (HMGB1) and exogenous (LPS) ligands of TLR4. These in turn activate production of ROS, neutrophil extracellular traps (NETs), and downstream activation of the inflammasome, producing inflammatory cytokines and other mediators that promote expression of adhesion receptors and ligands on endothelium and blood cells. Intravascular hemolysis also releases adenine nucleotides, including ATP and ADP, which further contributes to platelet activation. There is also some evidence that adenosine binds receptors on red cells, resulting in increased 2,3-diphosphoglycerate and sphingosine-1-phosphate, associated with lower oxygen affinity of hemoglobin (not shown). Proteins on the surface of the activated endothelium (P-selectin, E-selectin, VCAM1, ICAM1) interact with adhesive platelets, neutrophils, and sickle erythrocytes, producing vasoocclusion and acute chest syndrome. Intravascular hemolysis also releases asymmetric dimethylarginine, which inhibits eNOS. CRP, C-reactive protein; SAA, serum amyloid A; Orn, ornithine. Adapted with permission from Gladwin, et al., *Journal of Clinical Investigation* (150).

appreciated as an additional important mediator of inflammation and vascular injury (102, 103). In sickle cell mice, free heme drives inflammation, vaso-occlusion, and coagulation that are blocked by the heme scavenger hemopexin (104–109). In cultured cells, heme promotes secretion of high levels of placenta growth factor (110), which in turn induces release of the potent vasoconstrictor endothelin 1 (111), a common mediator of PH. Heme is a potent source of oxidant stress, but it does not scavenge NO.

Hemoglobin oxidation is driven not only by reaction with NO (112), but also by reaction with a host of additional physiological oxidants (113–118). Ferric and ferryl forms of hemoglobin produced in SCD and other forms of hemolysis are highly reactive in promoting oxidation (119–121). Hemolysis also produces red cell microparticles that can deliver toxic heme to endothelial cells (122, 123). Heme species appear to activate innate immune sterile inflammation pathways through TLR4 and NALP inflammasome signaling (104, 105, 124, 125). As such, these hemolysis products are proposed to represent erythrocyte danger-associated molecular patterns (eDAMPs), which promote and propagate sterile inflammatory and oxidative stress, further impairing the redox balance (126, 127). eDAMP release and oxidation of plasma hemoglobin

to methemoglobin, which is necessary for the release of heme, are likely enhanced during acute vaso-occlusive episodes. To date, a careful stoichiometric analysis of cell-free hemoglobin levels, heme levels, and heme in microparticles during sickle vaso-occlusion has not been performed. Heme, hemoglobin, and red cell ADP activate platelets and stimulate platelet mitochondria to produce ROS and cause an oxidative enzymopathy of complex V (the ATPase), resulting in platelet activation, thrombospondin-1 and PDGF release, and promotion of inflammation and vasculopathy (128). eDAMPs trigger innate immune responses, perhaps in the setting of LPS-priming of the NALP3 inflammasome, and might be central to the sterile inflammation that is characteristic of sickle vaso-occlusion.

In this context, we propose that steady-state intravascular hemolysis primarily inhibits NO signaling and amplifies ROS formation, tipping the redox balance and producing endothelial dysfunction (Figure 2). With advancing age, this effect drives development of vasculopathic complications that characterize the subphenotypes of SCD most closely associated with hemolysis. Steady-state inflammation and increased blood viscosity also promote cellular adhesion and vaso-occlusion, leading to acute painful episodes and organ ischemia/reperfusion injury. These mecha-

nisms intersect, perhaps during severe vaso-occlusive painful crisis when acute hemolysis is triggered and oxidant stress enhances hemoglobin oxidation and heme release. This intersection activates primed innate immune signaling pathways and the inflammasome, leading to multisystem injury and acute lung injury.

Controversies regarding the hyperhemolysis model

The role of hyperhemolysis as a proximate cause of some SCD complications has drawn criticism. In some cases, this was a dispute over nomenclature; in others, similar data were interpreted differently (11, 12). Importantly, the debate has not centered on reproducibility, as strong associations between morbidity and mortality and measures of cell-free plasma hemoglobin and other markers of hemolysis, TRV, and PH remain robust. Similarly, strong consensus surrounds the vasoactivity and injurious effects of hemolysate, cell-free hemoglobin, and heme. Some of these controversies actually represent consensus, and are summarized in the following objections and responses:

Echocardiography-defined TRV is not adequate to diagnose PH. This is clearly true. TRV is a physiological biomarker representing PH risk, much as transcranial Doppler velocity represents stroke risk in children with SCA. PH diagnosis requires pulmonary artery catheterization. TRV ≥ 3 m/s appears to have about 75% specificity for PH in adults with SCD (64, 65). TRV ≥ 2.5 m/s specificity is approximately 25% for PH, but the addition of abnormally short 6-minute walk distance (64) or elevated serum NT-proBNP (65) can enhance identification of high-risk patients in this intermediate TRV group. Elevated TRV unequivocally represents a higher risk of PH diagnosed by right heart catheterization (19, 64), but this can be confounded by high cardiac output, error in the estimation of TRV, and other sources of variability. TRV also represents an elevated risk of impaired exercise tolerance, proteinuria, venous thromboembolism, and mortality (Figure 1 and refs. 58, 129, 130).

PH does not occur in 34% of SCD adults. This is correct. Approximately 6%–10% of SCD adults have PH defined by a mean pulmonary artery pressure greater than or equal to 25 mmHg, measured by pulmonary artery catheterization (64–66). However, another 25% of patients have mildly elevated TRV, which is prognostically significant, as discussed above, and mean pressures between 20 and 25 mmHg are abnormally high and likely consequential.

PH in SCD is caused by left ventricular diastolic dysfunction. Several echocardiography and right heart catheterization studies have shown that half of PH cases in SCD involve precapillary PH consistent with inappropriately high pulmonary vascular resistance, leading to right ventricular hypertrophy and failure (64–66, 74, 131). The other half comprise postcapillary PH, associated with a stiff left ventricle due to ventricular hypertrophy and linked to anemia and chronically high cardiac output (64–66, 68, 74). Even in patients with high left atrial pressures, the risk of death most closely associates with increases in the intrinsic pulmonary vascular resistance (high pulmonary vascular resistance and transpulmonary pressure gradient). An additional mechanism of cardiomyocyte dropout and cardiomyopathy has been found in sickle mice (132).

Serum LDH is not a good biomarker of hemolysis. In large population studies, LDH values in homozygous SS patients are correlated with higher levels of more direct markers of intravascular hemolysis, cell-free plasma hemoglobin, and red cell-derived microparti-

cles (89). LDH values also correlate in human physiological studies with an impaired response to NO donor infusions. However, LDH has limitations as a marker of hemolysis, since it is released by lytic damage of almost any tissue, which occurs in patients with SCD. Its assay methodology varies among different clinical laboratories, complicating multicenter analyses. Serum LDH is a biomarker of intravascular hemolysis, which releases free hemoglobin and arginase. Both are integral to the hyperhemolysis model of NO scavenging (10). Phagocytosis of damaged red cells by macrophages or extravascular hemolysis is not expected to release free hemoglobin or LDH into plasma. Red cell survival studies do not distinguish between extravascular and intravascular hemolysis (133). Significant variability in serum LDH in steady-state SCD adults is provided by LDH isoforms originating from red cells but also found in renal cells (20). Until better biomarkers for intravascular hemolysis are available, only serum LDH, aspartate aminotransferase, and plasma hemoglobin can be used to imperfectly indicate intensity of intravascular hemolysis.

Decreased NO bioavailability cannot be the sole mechanism of vasculopathy in SCD. This is also correct. Published data evidence the involvement of oxidative stress (79, 100, 105, 108, 113, 115, 116, 121, 123, 132, 134, 135), inflammation (9, 22, 27, 32, 101, 105–108, 115, 124–127, 134), dyslipidemia (135–138), microparticles (89, 122, 123, 139), and vasoactive peptides (110, 111, 140–143). These additional pathways (depicted in Figure 2) are potentially additive or synergistic to intravascular hemolysis-like mechanisms. We note that hemolysis potently impairs redox balance, lowering NO signaling and enhancing pathological ROS signaling.

Markers of hemolysis do not correlate with red cell survival. A recent study limited to 13 measurements failed to find such correlations (133). This small study considered only 11 pediatric SCA patients with very low levels of basal hemolysis based on hydroxyurea treatment, many with α -thalassemia trait, and most having unusually high levels of HbF (10 of 13 measurements came from patients with HbF levels greater than 9%, including one of 33.8%). Measuring correlations of hemolysis markers in patients with limited hemolysis does not adequately test biomarkers. These studies should be performed in adult patients with clinically relevant ranges of hemolytic severity. Additionally, red cell survival (total hemolysis) in SCD is believed to be dominated by extravascular hemolysis (144), which is not the mechanism proposed to scavenge NO. Presently, plasma hemoglobin, serum LDH, and AST, with all their limitations, remain the best available biomarkers of intravascular hemolysis. Intravascular hemolysis must not be confused with extravascular hemolysis.

Conclusions

Epidemiological associations and mechanistic causal testing support the pathogenic role of intravascular hemolysis in SCD. New understanding of the role of red cell hemolysis products, redox disequilibrium, and eDAMPs in the end-organ injury observed in SCD provides a pathway for identifying counterregulatory signaling pathways that might dampen sterile inflammation and oxidative stress, e.g., upregulation and protective polymorphisms in the heme oxygenase-1 enzyme (134, 145). Upstream activation of the KEAP1/NRF2 redox sensing transcription pathway, a central counterregulatory program that protects against oxidative and hemolytic stress, is being actively investigated as a therapy for SCD (146,

147). As clinical trials are planned, it will be vital to target drugs to specific subphenotypes of SCD when considering treatments beyond the direct inhibition of deoxy-HbS polymerization. Given the results of the Gardos channel inhibitor clinical trial, new drugs that reduce hemolysis and increase hemoglobin levels should be used cautiously, possibly in combination with optimal dosing of hydroxyurea or other agents that directly inhibit HbS polymerization by inducing high levels of HbF, or another mechanism to limit viscosity, inflammation, and vaso-occlusion and its complications. Drugs that promote cGMP signaling, such as soluble guanylate cyclase stimulators, should be tested with caution, as the phosphodiesterase 5 inhibitor sildenafil induced painful crisis in clinical trials (148). Development of promising hemoglobin- and heme-scavenging agents is likely limited to more acute therapy in the setting of hyperhemolysis, akin to the use of eculizumab for hemolytic uremic syndrome. Common nodes that intersect both hemolysis-endothelial dysfunction and viscosity/vaso-occlusion, such as the KEAP1/NRF2 pathway and NALP3 inflammasome, might be ideal targets for therapy. Hydroxyurea is a prototype for such an agent, as it inhibits HbS polymerization via HbF induction and is metab-

olized in vivo to form NO (92, 149). Finally, intravascular hemolysis is surely not the only source of vasculopathy in SCD, and more vigorous research is needed on the role of oxidases, oxidant stress, eDAMPs, and the inflammasome pathway.

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