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.

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Intravascular hemolysis and the pathophysiology of sickle cell disease

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Intravascular 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 acidic 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.
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.

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

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

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, proteinuria 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 antimethemoglobin pharmacological intervention substantially phenocopy SCA–α-thalassemia, further supporting phenotypic modulation of SCD by hemolysis and anemia. Other than increased blood viscosity, only increased adhesivity 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 μM (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), CSSD (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), spheroctysis, 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 three 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).
Table 2. A comparison of SCD cohorts studied with right heart catheterization

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

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
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 extracellular 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 inappropriate 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 microparticles (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 isofoms 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, 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.

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