Basic Mechanisms of Hemolysis-Associated Thrombo-Inflammation and Immune Dysregulation

Jordan D Dimitrov, Lubka T Roumenina, Gina Perrella, Julie Rayes

Basic mechanisms of hemolysis-associated thrombo-inflammation and immune dysregulation during infection

Jordan D. Dimitrov[1*], Lubka T Roumenina[1], Gina Perrella[2], Julie Rayes[2*]

1. Centre de Recherche des Cordeliers, INSERM, CNRS, Sorbonne Université, Université de Paris, Centre de Recherche des Cordeliers 15, rue de l'Ecole de Médecine, F-75006 Paris, France.
2. Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Vincent Drive, B15 2TT, Birmingham, U.K.

*Corresponding authors: Dr Jordan Dimitrov, jordan.dimitrov@sorbonne-universite.fr & Dr Julie Rayes, J.rayes@bham.ac.uk

Keyworks: Hemolysis, Heme, Thrombosis, Infection, Inflammation

Abstract

Independent of etiology, hemolytic diseases are associated with thrombosis, inflammation and immune dysregulation, all together contributing to organ damage and poor outcome. Beyond anemia and the loss of the anti-inflammatory functions of red cells, hemolysis leads to the release of damage associated-molecular patterns (DAMPs) including adenosine diphosphate, hemoglobin and heme which act through multiple receptors and pathways leading to a hyperinflammatory and hypercoagulable state. The oxidative, inflammatory and thrombotic environment is fostered by a cooperation between platelets, endothelial and innate immune cells as well as the coagulation and complement cascades. Extracellular free heme is a promiscuous DAMP which interacts with different receptors and plasma proteins, orchestrating a complex oxido-inflammatory environment leading to thrombosis. In this review, we discuss the main mechanisms by which hemolysis and in particular heme, drive this thrombo-inflammatory milieu. We finally discuss the key drivers of hemolysis-associated immune dysregulation and its contribution to secondary complications during infection.

Introduction

Hemolysis is a pathological process characterized by the premature loss of the membrane integrity of red blood cells (RBCs) leading to the release of the cytosolic content, mainly comprised of hemoglobin, in the extracellular space. It can be triggered by various pathological factors including genetic abnormalities of hemoglobin (such as in sickle cell disease (SCD), β-thalassemia, etc.) or complement regulators (as in atypical hemolytic uremic syndrome, paroxysmal nocturnal hemoglobinuria (PNH), pathogens (as observed in malaria, sepsis, typical hemolytic uremic syndrome, etc.), auto- or alloantibodies, oxidative stress, toxins, trauma or blood transfusion. In this review, we will focus on the functions of hemoglobin and heme as DAMPs and their direct and indirect contributions to inflammation, thrombosis and immune dysregulation.

Molecular structure, conventional physiological functions and unconventional activities of hemoglobin and heme

Heme is a coordination complex of the macrocyclic component protoporphyrin IX and iron ion. Protoporphyrin IX consists of four pyrrole rings, derivatized with propionate, vinyl and methyl groups (Figure 1A). The nitrogen atoms of pyrroles in heme occupy four out of the six total coordination sites of iron ion, leaving two coordination sites available for interaction with other ligands[1]. Heme is used as a cofactor molecule in a vast number of proteins implicated in aerobic metabolism of both prokaryotic and eukaryotic cells. As a prosthetic group of hemoproteins, heme has two main features: first, it has an extraordinary capacity to fine-tune its functional activity depending on the amino acid residues that interact with the porphyrin structure while coordinating

Fe ion, potentially explaining the versatile functional activities of hemoproteins. Second, all hemoproteins are compartmentalized and never present in the extracellular space under physiological conditions[1]. The most prominent hemoprotein in vertebrates is hemoglobin, where heme is used for transient binding and transport of gaseous molecules, such as oxygen and carbon dioxide[2] (Figure 1B). The ability of heme's iron to easily alternates its oxidation state and to interact with multiple hemoproteins and receptors determines its functional activities. Indeed, heme is indispensable for the structural integrity and function of hemoproteins through high affinity or/and even covalent interactions as for example for heme *c* in cytochrome c. Heme is also able to bind to intracellular proteins in a transient manner. These proteins cannot be classified per se as hemoproteins as protein integrity do not depend on heme's binding. This is probably related to intrinsic binding promiscuity of heme, determined by its unique physiochemical molecular features (simultaneous presence of aromatic, hydrophobic, charge and metal coordination interacting moieties) (Figure 1A). Despite transient interactions, disruption of heme binding to selective proteins shows profound functional consequences. Not surprisingly, heme was demonstrated to directly participate in pathology and tissue damage accompanying hemolytic diseases with different etiology and is suggested as a promising drug target to limit both thrombosis and inflammation.

The scavenging system during hemolysis

The prominent toxic effects of extracellular hemoglobin and heme well justify the existence of evolutionary conserved system for scavenging and disposal of these by-products of hemolysis. The two plasma proteins haptoglobin and hemopexin are specialized for binding, neutralization of toxic effects, and safe disposal of hemoglobin

and heme, respectively[3, 4]. These proteins interact with their ligands with very high binding affinity and transport them for degradation by macrophages in the spleen and liver. The hemoglobin-haptoglobin complex is recognized by the scavenger receptor CD163, whereas heme-hemopexin complex is recognized by CD91, also known as low-density lipoprotein receptor-related protein 1 (LRP1). As a consequence of its high affinity to heme (K_D value < 10^{-10}), hemopexin can be consider as a plasma protein that irreversibly binds heme[3, 4]. Nevertheless, plasma contains many other proteins and macromolecules that can interact with heme, albeit with lower affinity. These proteins include the most abundant plasma proteins albumin[5] and immunoglobulins (Ig)[6], as well as proteins present at lower concentration such as alpha-1 microglobulin[7], C1q[8], C3[9], Factor VIII[10], fibrinogen[11] etc. Delivery of hemolytic products to macrophages engages an intracellular system for their safe disposal involving heme oxygenase-1 (HO-1) and ferritin. The former enzyme is responsible for the oxidative degradation of heme to biliverdin, ferrous ions and carbon monoxide (CO)[12]. The induction of HO-1 and ferritin overexpression is not dependent on a specific membrane receptor and is regulated by intracellular heme levels through multiple transcription factors including nuclear factor erythroid 2-related factor 2, activator protein-1, Bach1 and hypoxia-inducible factor[13]. However, extensive hemolysis overwhelms the scavenging system and as a consequence, the pathological potential of extracellular hemoglobin and heme can be manifested.

Hemoglobin and heme oxidative functions

Beyond anemia, hemolysis is associated with the depletion of nitric oxide (NO) as a result of L-arginine deficiency (required for NO synthesis) and the scavenging of NO by hemoglobin resulting in the disruption of vascular homeostasis [14]. Moreover, the potent pro-oxidant nature of extracellular hemoglobin and heme catalyzes oxidative

reactions generating reactive oxygen species (ROS) that can oxidize vulnerable macromolecules[15]. These modified molecules can after serve as ligands for innate danger-sensing receptors, triggering pro-inflammatory programs. For example, heme was shown to oxidize LDL which can subsequently activate TLR2 and TLR4 promoting pathogenic innate immune cells activation[16, 17]. Additionally, free heme catalyzes the formation of hydroxyl radicals in the Fenton reaction through the redox-active iron and induces lipid peroxidation, increasing cell membrane damage, permeability and death[15]. Consequently, hemolysis-associated reduction in the scavenging of heme and hemoglobin, the release DAMPs and reduced NO availability exacerbate thrombo-inflammation.

Pro-inflammatory and immune dysregulation activities of heme

Heme-induced activation of innate immune receptors and pathways

In 2004, Seong and Matzinger presented a hypothesis that hydrophobic molecules (or hydrophobic patches on macromolecules) activate innate immune cells by binding to specific pattern recognition receptors (PRR), a mechanism which unifies the immune-stimulatory effects of DAMPs and PAMPs on innate cells[18]. Heme is a highly hydrophobic molecule and, as such, fits perfectly with the definition of Matzinger for a DAMPs[19]. Indeed, Soares and Bozza classified also heme as an alarmin, an endogenous molecule that, when released extracellularly, induces cellular damage by interacting with multiple receptors on different cells[19]. This picture would be even further complicated when hemolysis is secondary to an infection where DAMPs and pathogen-associated molecular patters (PAMPs) synergistically activate pro-inflammatory and prothrombotic pathways (Figure 2).

Effect of heme on monocytes and macrophages

Heme has pleiotropic effects on neutrophils, monocytes and macrophages which are specialized in the protection against pathogen (reviewed in[20-22]). An alteration in both classical and non-classical (patrolling) monocytes was observed in patients with SCD and PNH. Indeed, the number of HO-1^{high} patrolling monocytes is significantly decreased in patients with SCD following a recent VOC episode and a deficiency in these monocytes exacerbates VOC in SCD mice [23]. From the other hand, the level of classical highly inflamed monocytes is increased in SCD and PNH patients and engulfment of hemoglobin-activated platelets by classical monocytes increases their inflammatory phenotype and death *in vitro*[24]. Moreover, cell-free hemoglobin S but not heme increases the expression of inflammatory cytokines in human monocytes in a TLR4/MD2-dependent manner[25].

In a seminal study, Figueiredo *et al.* demonstrated that heme serves as ligand for TLR4[26]. By binding to TLR4 on macrophages at a distinct site to the one for lipopolysaccharide (LPS), heme increases the expression of TNFα and cell activation, albeit the response is partial as compared to LPS[26]. The synergy between the oxidative burst and TNFα production triggers necrosis in macrophages[27]. Mechanistically, heme activates NF-kb, however the details about the interaction of TLR4 with heme are still controversial[28-31]. *In vivo* experiments inhibiting TLR4 signaling or TLR4 deficient animals emphasized the pathological importance of TLR4-heme interaction in various disease contexts including SCD[32-39]. Similarly, heme polymer hemozoin found in blood feeding parasites, such as *Plasmodium falciparum,* activates innate immune cells (dendritic cells) through TLR9. Importantly, injection of hemozoin into mice results in the systemic release of pro-inflammatory cytokines, an effect that was abrogated in TLR9 knockout mice and in mice deficient in MyD88[40]. In

addition to TLR pathways, heme engages and activates ROS-dependent signaling as well NLRP3 inflammasome in macrophages, independent of heme internalization and activation of the purinergic receptors[31, 41]. In the absence of macrophage priming by LPS, activation of caspase-4 and caspase-5 is required for the release of pro-inflammatory cytokines[42]. In LPS-primed macrophages, heme-induced NLRP3 activation promotes the cleavage of IL-1β in a spleen tyrosine kinase (syk), NADPH oxidase-2 and mitochondrial ROS and K(+) influx-dependent manner and contributes to the lethality during sterile hemolysis[43].

Beside macrophage activation, heme promotes the differentiation of monocytes to a tolerogenic macrophage subtype specialized in the phagocytosis and the disposal of erythrocytes and their debris[44, 45]. In SCD, extracellular heme induces the production of type I IFNs by monocytes and macrophages leading to the recruitment and differentiation of monocytes to a specific population of macrophages in the liver which expresses high level of Fc-γRIa (CD64) which efficiently binds to IgG-opsonized targets, exacerbating anemia through IgG-mediated phagocytosis [46, 47]. In this context, it is important to note that heme has also the capacity to induce autoreactivity and polyreactivity in a fraction of IgG antibodies present in healthy humans[6, 48-50]. Heme binds to the variable region of antibodies and allows antibodies to recognize unrelated proteins with relatively high binding affinity. Whether heme-induced polyreactive IgGs activates Fc-γ receptors on innate immune cells and platelets and propagates immunothrombosis and thrombo-inflammation requires further investigation. In addition, heme impairs macrophage phagocytic and chemotactic functions[22, 51] altering actin polymerization and disrupting the cytoskeleton dynamics. This effect is mediated by high affinity binding of heme to DOCK8 resulting in the activation of small Rho GTPase CDC42 which regulates actin dynamics. Moreover, excessive RBCs

phagocytosis by hepatic macrophages was shown to induce an immunosuppressive phenotype in the liver during sepsis leading to bacterial growth and to a decrease in survival[52]. In *Klebsiella pneumoniae* infection, heme suppresses STAT-1 and interferon responses in the liver independent of HO-1 and the iron ion but through the porphyrin[52]. This data show that heme, via different moieties, alters macrophage functions and impairs bacterial clearance, exacerbating sepsis.

Beside TLR4, heme binds to RAGE and induces its oligomerization, leading to activation of cell signaling, phosphorylation of ERK 1/2 and AKT, and to the release of the pro-inflammatory cytokines IL-1β, TNFα and IL-6 in the lung *in vivo* [53]. The promiscuous expression of RAGE and TLR4 on immune and endothelial cells (ECs) as well as platelets might explain the systemic consequences associated with heme release. However, the expression of heme-binding receptors in a specific vascular bed such as RAGE in the lung or increased susceptibility to specific ECs as observed in glomerular ECs could increase toxicity of heme and its thrombo-inflammatory effects in selective organs[53, 54].

Effect of heme on neutrophils

In 2004, Graça-Sousa *et al*. showed that exposure of human mature neutrophils to heme induces protein kinase C activation, IL-8 expression, oxidative burst, the expression of adhesion molecules and increased chemotactic activity of cells towards heme gradients[55, 56]. Importantly, the chemotactic activity and secretion of IL-8 induced by heme were preserved in the presence of human serum proteins. Heme induces MAPK, PI3K/Akt and NF-κB activation, with ultimate anti-apoptotic effect[57], subsequently increasing their survival and fostering an inflammatory environment. Heme-induced oxidative burst in neutrophils is not solely dependent on TLR4 but also

involves G protein-coupled receptors[58]. On the other hand, heme was shown to induce the expression of HO-1 in immature neutrophils in the bone marrow and to support their mobilization in the bloodstream[59]. These immature neutrophils have a defect in oxidative burst activity resulting from impaired ROS signaling without a significant effect on mature neutrophils[59]. Beside altering neutrophil chemotaxis and oxidative burst, heme induces neutrophil extracellular trap (NET) formation in TNFα-primed neutrophils and heme-mediated NET release contributes to vaso-occlusive crisis (VOC) in SCD [60, 61]. Interestingly, whereas heme does not induce NETosis in non-primed neutrophils[60], addition of platelets induces potent NETosis[62], supporting that a cooperation between platelets and neutrophils can drive thrombosis in hemolytic conditions.

Interaction of heme with the complement system

The complement cascade is an integral part of the innate immune system comprised of plasma and membrane-bound proteins essential for host defense against pathogens (predominantly bacteria) and in sustaining tissue homeostasis[63]. Despite its protective functions, dysregulated activation of the complement system exerts potent tissue-destructive and pro-inflammatory functions. Free heme was shown to interact with the central complement component C3 and to activate the alternative pathway of the complement cascade[9], while its binding to C1q inhibits the classical pathway[8]. Moreover, heme interacts with the complement regulator Factor I and perturbs the negative regulation of the cascade, amplifying complement activation, release of anaphylatoxins and tissue damage[64]. Altogether, heme causes a net overactivation of complement, which was suggested to amplify erythrocyte destruction in malaria[65, 66]. Indeed, even in the case of low parasite burden, patients may develop

severe anemia as a result of heme-mediated complement activation and the clearance of CD35-positive young erythrocytes opsonized by C3b. Therefore, prophylactic complement inhibition at the level of C3 for malaria-infected individuals may be a potential therapeutic strategy to reduce the risk for anemia[66]. Beside inducing a prothrombotic phenotype in ECs, heme-induced P-selectin expression serves as a platform for attraction of C3 activation fragments and complement overactivation on the endothelial surface[36] contributing to the pathology of hemolytic diseases[67, 68], infections or in rhabdomyolysis-induced acute kidney injury or transfusion reactions[9, 68-71]. Interaction of heme with complement proteins in the context of bacterial infections can have different detrimental consequences. First, the capacity of heme to inhibits the binding of C1q to IgG and IgM[8, 72] might alter the activation of the classical pathway by the microbes opsonized by antibodies. Second, direct activation of C3 by heme in a systemic manner might deplete it, thus temporally diminishing the potential of complement to counteract bacterial infection. Thirdly, the release of anaphylatoxins following uncontrolled activation of alternative pathway of complement by heme activates different immune cells in a systemic manner, thus causing immune exhaustion and deterioration of their specific immune defense activity.

Prothrombotic effects of hemolysis, hemoglobin and heme

It is well established that hemolysis contributes to a hypercoagulable state. NO depletion during hemolysis is considered a key driver of oxidative stress and thrombosis during hemolysis. Hemoglobin released during hemolysis scavenges NO at a rate 1000 times more effectively than that encapsulated in the RBC contributing to vasoconstriction and hypercoagulation[73]. Inactivation of plasma hemoglobin by oxidation or NO ligation restore NO bioavailability. Hemolysis is associated with

pulmonary arterial hypertension, kidney injury and increased risk of ischemic complications. In patients with SCD, the exposure of phosphatidylserine (PS) on RBC membrane and microvesicles (MVs) contributes to VOC and to progressive organ damage [74].

Endothelial cells as a target of hemolysis

ECs are considered the first and main target of hemolysis-associated DAMPs. Interestingly, some ECs have high phagocytic capacity and can engulf damaged RBCs and clear them from the circulation[75]. ECs phagocytose clots and clear emboli from the microvasculature through hemodynamic pressure and translocation of these clots to the perivascular space, a mechanism named angiophagy [76]. Brain ECs have high capacity of erythrophagocytosis of damaged RBCs exposed to oxidative stress compared to healthy cells associated with the passage of hemoglobin across the monolayer of ECs. This passage of pro-oxidative hemoglobin through the brain barrier was proposed to contribute to cerebral microbleeds in the absence of endothelial disruption[77].

EC activation by hemoglobin and heme increases the expression of adhesion and pro-thrombotic molecules supporting immune cell and platelet adhesion[78, 79]. Hemoglobin induces NF-kb activation and hypoxia inducible factor, increases ROS generation and cell permeability[80]. This effect was attenuated by blocking MyD88 and NF-kb but not TLR4[80]. It also increases TF expression on ECs in response to LPS showing synergy between infectious agents and hemolysis to induce TF by EC[78]. Similar to hemoglobin, heme induces EC activation and triggers VOC in an experimental SCD in a TLR4-dependent manner [33]. Indeed, TLR4 expression on endothelial rather than on hematopoietic cells was shown to initiate VOC in SCD mice[38]. Heme induces the expression of adhesion molecules (ICAM-1, VCAM-1, P-selectin), TF expression and

the exocytosis of Weibel-Palade bodies associated with the release of VWF[33, 36, 79, 81]. Whereas multiple studies showed the upregulation of TF on ECs *in vitro*, the expression and role of endothelial TF is not well established. Indeed, using mild and severe SCD experimental models, *Solovey et al* showed that TF expression is mostly observed in the pulmonary veins[82]. Subjecting mice to hypoxia/regeneration increases TF on lung endothelial cells in SCD but not in WT mice[82]. However, *Sparkenbaugh et al* showed that heme injection induces TF on immune cells, both neutrophils and monocytes, but not on endothelial cells[81]. TF was also observed in the perivascular space and heme-induced coagulation depends on the expression of TF on both hematopoietic and non-hematopoietic cells. The importance of P-selectin in VOC was shown using P-selectin-deficient mice and by using anti-P-selectin blocking antibodies. Indeed, anti-P-selectin antibody Crizanlizumab (ADAKVEO) reduces the rate of pain crises, adverse effects, and decreases the frequency of VOC in SCD patients[83]. Crizanlizumab-tmca (ADAKVEO) was FDA-approved in 2019 to decrease the frequency of VOC in adults and pediatric patients aged 16 years and older with SCD. The effect of anti-P-selectin is likely multifactorial by limiting immune cell adhesion on ECs and also formation of platelet-leukocyte aggregates, thus decreasing inflammation and thrombosis[65]. However, it is important to note that long-term P-selectin deficiency was shown to reduce immune cell recruitment to the liver increasing cellular senescence and reducing epithelial cell proliferation, thereby impairing the resolution of inflammation in SCD mice [84]. Therefore, fine-tuning of inhibition of immune cells and their effect on the resolution of inflammation during hemolysis is required to limit organ damage, independent of VOC.

More recently, heme but not hemoglobin was shown to activate the NLRP3 inflammasome in ECs and to stimulate the release of IL-1β[85]. This activation depends

on heme-induced ROS generation and requires the structural integrity of heme as neither protoporphyrin IX nor Fe ions alone induced IL-1β production. The lipophilic nature of heme also promotes increased incorporation in the plasma membrane, rising cell sensitivity to oxidative stress. Indeed, heme induces mitochondrial dysfunction, activates autophagy and induces lipid peroxidation[86]. Not only free heme is toxic to ECs, but also RBC-derived MVs are able to bind to ECs and to transfer heme, triggering ROS generation and VOC in SCD[87]. The pro-oxidative and prothrombotic effects of these MVs are neutralized by hemopexin, by blocking PS using annexin-V or by inhibiting TLR4[87]. Importantly, the downstream effect of heme is dose-dependent as high concentrations of heme induce apoptosis and necroptosis in a TLR4 and ROS-dependent manner[87, 88]. Beside direct activation by heme, endothelial cells are prone to complement attack triggered by heme with increased susceptibility to activation in selective vascular bed. Heme increases complement C3 deposition on glomerular ECs and this effect was associated with lower complement regulator factor H binding and a defect in thrombomodulin upregulation[54]. In a model of experimental sterile hemolysis induced by phenylhydrazine, P-selectin was also shown to drive complement attack on ECs in a TLR4- and heme-dependent manner[36]. Heme also induces VWF release, regulating complement activation and platelet recruitment during hemolysis. Thus, by binding to complement factor H, VWF acts as a negative regulator of complement activation increasing factor H-mediated C3b inactivation and reducing complement attack[89]. This effect is regulated by the size of VWF multimers as ultra-large multimers lack the cofactor activity and the negative regulation of complement activation[90]. This effect was confirmed using blood outgrowth endothelial cells isolated from patients with von Willebrand disease type 3 showing increased susceptibility to complement deposition and cytotoxicity compared to healthy ECs [91].

Therefore, heme-induced complement activation and susceptibility in specific vascular bed confers selectivity to innate immunity-driven thrombosis and supports the development of distinct thrombo-inflammatory mechanisms in different organs. This can be enhanced by the specificity of endothelial cells in erythrophagocytosis, susceptibility to complement activation, TLR4 expression and protein content.

Hemolysis-induced platelet activation: the central role of hemoglobin and heme in thrombosis

During hemolysis, platelet activation and platelet-leukocyte complexes were shown to contribute to thrombo-inflammation. The release of ADP from damaged cells can induce platelet aggregation through the activation of $P2Y_1$ and $P2Y_{12}$. Indeed, injection of hemolysate induces platelet aggregation, whereas platelets from patients treated with $P2Y_{12}$ inhibitors showed reduced platelet activation by RBC lysates[92]. Platelet activation-mediated P-selectin expression was also shown to support platelet-neutrophil complexes increasing neutrophil activation and their oxidative activity and potentiating lung vascular permeability[93]. Targeting P-selectin or $P2Y_{12}$ reduces platelet-neutrophil aggregates and improved lung vascular permeability in SCD mice[93]. Using intravital microscopy, ADP released during hemolysis was shown to induce platelet-rich thrombi in the pre-capillaries pulmonary arterioles in GPIIb/IIIa- and $P2Y_{12}$-dependent manner, suggesting that targeting ADP in hemolytic diseases could decrease thrombotic complications[94]. However, the data from the DOVE multinational trial assessing the efficacy of $P2Y_{12}$ inhibitor, Prasugrel, in patients with SCD showed no amelioration in the rate of VOC compared to placebo[95], suggesting the existence of multiple mechanisms by which hemolysis-associated DAMPs can regulate ischemic events.

Beyond their pro-oxidant functions, hemoglobin and heme trigger platelet aggregation through different mechanisms in a dose-dependent manner[62, 96-98]. Indeed, it was shown that at low concentrations of hemoglobin induces platelet activation and aggregation *in vitro* through GPIb-α whereas at high concentrations it induces platelet apoptosis[99]. However, another study showed that hemoglobin does not induce direct platelet activation but rather abrogates the effect of NO on platelets [99]. This study showed that hemoglobin-bound ADP was responsible for the direct platelet activation, which was inhibited by apyrase. Beyond platelet activation, hemoglobin interacts with VWF (via the A1 domain), cooperates with GPIb-α to increase VWF-mediated platelet aggregation and microthrombi formation on fibrinogen and collagen at high shear rate[100]. Inhibition of hemoglobin binding to GPIb-α decreases thrombus formation on VWF and collagen[100]. Hemoglobin also binds to the A2 domain of VWF and blocks its cleavage by ADAMTS13, facilitating thrombus formation[101].

Not only hemoglobin is able to activate platelets, but free heme was also shown to be even a more potent activator of platelets[97]. In cases of hemolysis and release of heme, it is unlikely that heme will be present in monomeric “soluble” form for binding to the receptors but most probably heme will be bound to certain plasma macromolecule with low affinity. Moreover, it is important to note that heme, when not bound to proteins, has a high tendency to form dimers and oligomers[102]. These aggregates of heme may have potent immune-stimulatory effects due to the crosslinking of receptors. The formation of oligomers might account for the toxic effect of heme on platelets, leading to damage and death of these cells[96, 98]. Indeed, based on a number of studies, *Hopp and Imhof* discussed the pivotal role for heme concentrations in bleeding and thrombotic complications, whereby high concentrations of heme induce bleeding while low concentrations induce hypercoagulopathy[103]. Beside triggering coagulation, heme

alters the activity of different coagulation proteins. Thus, heme binds to FVIII and impairs its interaction with FIX leading to a defective procoagulant activity[10]. Heme also increases FXII-dependent coagulation and it was shown to inhibit activated protein C anti-coagulant but not cytotoxic functions and to impair clot lysis[104]. Heme, through its iron ion and the protoporphyrin ring, binds and crosslink fibrinogen and inhibits the conversion to fibrin. Moreover, RBC lysate was shown to increase tissue plasminogen activator (tPA)-mediated fibrinolysis in whole blood whereas platelet lysate inhibits this process, highlighting the complexity of balancing thrombotic and bleeding complications associated with cell damage[105]. Fibrinogen also binds to hemozoin and this interaction activates monocytes through TLR4 and macrophage-1 antigen (MAC-1)[11, 106]. The dual effect of heme on coagulation factors and their activity can regulate the bleeding and thrombotic complications associated with hemolytic conditions [103].

Through its pro-oxidant functions, heme induces ROS generation, ferroptosis and inflammasome activation in platelets and these effects were inhibited by melatonin[107]. Interestingly, heme-induced platelet activation is independent of TLR4 but is mediated by the immunoreceptor tyrosine-based activation motif (ITAM) receptor CLEC-2[96]. Moreover, GPVI was also shown to contribute to platelet activation by heme and CLEC-2 and GPVI deficiency improves rhabdomyolysis-induced acute kidney injury in mice[108]. Indeed, CLEC-2 is activated by podoplanin expressed outside the vasculature whereas GPVI is activated by large number of agonists including collagen and fibrin and these studies identify heme as a novel agonist for CLEC-2 and GPVI[109]. Whether the level of expression of these receptors on platelets contributes to heme-induced platelet aggregation requires further investigation.

Platelet activation by heme was inhibited by Src, Syk or Btk inhibitors confirming the contribution of ITAM receptors in this process[96]. Importantly, platelet activation by heme was not altered by inhibiting $P2Y_{12}$ and COX-1, suggesting that heme-induced platelet activation is refractory to anti-platelet drugs[96]. Despite being dispensable for classical platelet activation by heme, TLR4 contributes mitochondrial ROS production and the release of thrombospondin-1 from platelets[97]. Interestingly, platelet aggregation induced by high concentrations of heme was not inhibited by ITAM signaling inhibitors or by blocking GPIIb/IIIa, suggesting a mechanism involving the agglutination of damaged platelets[96]. This could be further amplified by the interaction of fibrinogen with heme which opsonizes platelets and can contribute to drug-resistant platelet agglutination[106]. Moreover, heme-activated platelets induce NETosis and macrophage extracellular trap (MET) formation[110]. In this context, MET formation required GPIb-α-MAC-1 interaction and ROS generation and this mechanism contributes to kidney injury in a preclinical model of rhabdomyolysis[110]. The prothrombotic effect of heme could be counteracted by the expression of HO-1, limiting platelet aggregation and thrombus formation highlighting the importance of compensatory mechanisms to limit heme-mediated thrombosis [111].

Interestingly, the contribution of heme to thrombosis is not restricted to severe hemolytic diseases. An increase in hemoglobin and heme release was observed in patients with acute coronary syndrome and in infants and neonates undergoing cardiac surgery with cardiopulmonary bypass and high levels of free heme were associated with an elevated risk of thrombosis[112, 113]. Indeed, deficiency in HO-1 increases endothelial cell apoptosis, TF and VWF expression, platelet adhesion and favors ferric-chloride induced arterial thrombosis[114]. In this model, local hemolysis and hemoglobin oxidation were shown to contribute to the thrombosis, in the absence of

an overwhelming hemolysis[115]. Moreover, heme release from local hemolysis was shown to contribute to deep vein thrombosis (DVT). In a mouse model of DVT, a relatively high concentrations of heme peaked prior to clot formation and these levels diminished with thrombus resolution[116]. The role of heme in thrombus formation in DVT was confirmed using hemopexin-deficient mice. It was suggested that heme release might be secondary to the hemolysis of trapped erythrocytes and their oxidation. Interestingly, CLEC-2, the main receptor for heme on platelets is essential for clot formation during DVT, a mechanism suggested to be triggered by its interaction with podoplanin expressed in the vessel wall[117]. In this context, it is tempting to speculate that CLEC-2-podoplanin interaction could initiate DVT while CLEC-2-heme interaction supports thrombus growth.

Anti- and pro-inflammatory functions of RBCs: implications in hemolytic anemias and infections

Beyond oxygen transport, RBCs possess crucial anti-inflammatory functions limiting organ damage during sterile inflammation and infection. Indeed, RBCs can capture cytokines and chemokines from the blood, restraining their availability. The duffy antigen receptor for chemokines (DARC) is the main receptor for cytokines and chemokines on RBCs. It binds with high affinity to CC and CXC-chemokines, leading to their sequestration without supporting classical chemokine-induced cell responses[118]. Indeed, the lack of expression of the duffy gene on RBCs in patients with SCD was associated with chronic organ damage, in particular kidney dysfunction[119]. Beside binding chemokines, DARC is the key receptor mediating *Plasmodium vivax* infection of RBCs[120]. Individuals lacking DARC expression on RBCs are resistant to Plasmodium vivax malaria[120]. DARC is also a target for bacteria such as *Staphylococcus aureus* inducing hemolysis [39]. However, DARC is not

selectively expressed on RBCs but is also expressed on endothelial cells where it mediates the transcytosis of cytokines and chemokines from the inflamed basolateral area to the luminal surface, leading to their accumulation and increasing leukocyte adhesion and transmigration[121]. Therefore, DARC expression on endothelial cells supports inflammation, whereas RBC DARC acts as a sink for chemokines [122], showing that the spatial distribution of DARC on red and endothelial cells play a dual role in propagating and resolving inflammation.

RBCs are also important immune sensors for nucleic acids which can positively and negatively regulate organ function during homeostasis and inflammation, respectively. RBCs express TLR9 which binds to cell free CpG-containing DNA released from mitochondria (cf-mtDNA)[123] and from bacteria and viruses. During homeostatic conditions, *Hotz et al* showed that most cf-mtDNA is bound to RBCs through TLR-9 and this sequestration limits CpG-DNA-induced lung injury[123]. The protective function of RBCs is lost under pathogenic conditions, which can exacerbate inflammation and organ damage. Indeed, using a co-culture system, it was shown that RBCs isolated from healthy donors but not from trauma patients are able to bind CpG-DNA and to limit endothelial cell activation[123]. Under infectious condition, such as sepsis, CpG-DNA derived from pathogens binding to RBCs induces erythrophagocytosis, anemia and initiates local and systemic inflammation[124]. The pro-inflammatory environment is supported by the release of DAMPs from RBCs, in particular hemoglobin and heme, leading to inflammation, immune-dysregulation and thrombosis.

Consequence of release of heme for bacterial infections

As discusses above, the broad effects of heme on the innate immune components – complement, neutrophils, macrophages as well as endothelial cells and platelets

results in an overwhelming prothrombotic, pro-oxidative and pro-inflammatory response associated with a profound dysregulation in the defense mechanisms. This can lead to tissue damage and impaired host defense against pathogens. Beyond its ability to dysregulate or inhibit diverse functions of innate immune cells or complement proteins, heme contributes to bacterial infection as a growth factor. Indeed, many human pathogenic bacteria use heme as a source of iron for sustaining their proliferation[125]. Consequently, hemolysis and release of large quantities of heme can foster infection with pathogenic bacteria in two independent ways.

In addition, one of the co-morbidities and main cause of children mortality in patients with SCD are bacterial infections[127]. Indeed, hemolysis increases the susceptibility of patients with SCD and malaria to infection with *Salmonella Tiphimurium (S. Tiphimurium)*. In the absence of hemolysis, the spleen and liver are major sites for salmonella replication, however, in the context of hemolysis, bacteria infect neutrophils and impair oxidative burst along with a dysfunction in macrophage function[128]. Co-administration of heme with *S. Tiphimurium* induces the release of immature neutrophils from the bone marrow with defective oxidative burst and increases fatal bacteremia. In malaria, hemolysis impairs neutrophil oxidative burst through the induction of HO-1 and this effect could be reversed by inhibiting HO-1 by tin protoporphyrin[128]. Indeed, HO-1 induces tolerance to the toxic effect of heme but reduces ROS generation, thus balancing the resistance to pathogens. A defect in neutrophil oxidative burst activity was also observed in children with SCD, which is related to hemolysis and HO-1 induction in neutrophils[59]. Moreover, heme was shown to decrease CD4+ T cell proliferation and to significantly increase the frequency of regulators T cells (Tregs), an immunomodulator function mediated by heme/HO-1 axis and the presence of CD16+ monocytes [129]. Therefore, a mechanism of tolerance to

one infection might impair the resistance to another one. It should also be noted that heme has capacity to dysregulate the functions of B cells and to impair antibody responses as demonstrated in vitro and after intraperitoneal administration of heme in mice[130]. Collectively, these data suggest that heme can impair both innate and adaptive immune response against pathogens.

Hemolysis-associated immune dysregulation is not limited to SCD or malaria but could also be observed during sepsis. High level of cell free hemoglobin is associated with increased mortality in septic patients[131, 132]. Using experimental model of sepsis, Larsen *et al.* showed that high grade sepsis induces hemolysis whereas low grade sepsis did not[126]. The severity of sepsis was associated with high heme levels and a reduction in hemopexin levels[126]. Although heme administration to WT mice without sepsis did not induce lethality, its administration after low grade infection induces tissue damage and increases lethality in mice. Moreover, injection of heme with sublethal dose of heat killed bacteria induces 100% mortality compared to the low mortality in the absence of heme. Collectively, these data show the pivotal role of heme not only in regulating thrombosis, oxidative stress and inflammation but also its high capacity to dysregulate innate and adaptive immune response and promote tissue injury allowing the proliferation of pathogenic bacteria.

Conclusions

Independent of etiology, hemolysis drives thrombo-inflammation and oxidative stress contributing to organ damage. The promiscuous nature of heme and its possible interaction with multiple proteins and receptors in different organs along concentration-dependent functions suggest the development of distinct systemic and local mechanisms. Indeed, at the site of intravascular localized hemolysis, the level of free

heme can exceed the concentrations measured in the plasma. Results from multiple studies showing the efficacy of hemopexin in reducing thrombotic complications both in hemolytic diseases such as SCD but also in DVT suggest that targeting heme can be beneficial in a large number of diseases associated with hemolysis. Other strategies targeting platelet activation by heme such as inhibitors downstream ITAM receptors and TLR4 might also open new opportunities for treatment of complex and heterogenous thrombo-inflammatory mechanisms during hemolytic diseases. The effect of targeting heme will not only limit ischemic events, but can also reduce the severity of secondary infections in patients with comorbidities. Future work assessing the efficacy of targeting heme to reduce the severity of bacterial infections in DVT, coronary artery disease and myocardial infarction might open new therapeutic opportunities to address the detrimental effect of infectious in patients with comorbidities.

Acknowledgements: JR holds a British Heart Foundation (BHF) Intermediate Fellowship (FS/IBSRF/20/25039). GP is supported by a BHF project grant (PG/21/10737) for JR.

Conflict of interest: The authors have no conflict of interest to declare.

Author contributions

JDD and JR wrote the manuscript. LLTR and GP contributed to manuscript writing.

Figure 1: The structure of heme and hemoglobin. (A) Structural formula of iron protoporphyrin IX (heme b). The heme molecule has extraordinary binding promiscuity towards different proteins and other biomolecules. This stems from the presence in heme of groups capable of establishing various types of non-covalent interactions. The different types of interaction moieties of heme molecule are highlighted on the structural formula. **(B)** The structure of human hemoglobin. The

polypeptide chains of hemoglobin tetramer are depicted in blue. Heme is depicted in red. The figure was generated by UCSF Chimera software v. 1.16 using data from cryoelectron microscopy, PBD file 7XGY, downloaded from RCSB Protein Data Bank (https://www.rcsb.org).

Figure 2: Hemoglobin- and heme-induced oxidative stress, thrombosis, inflammation and immune dysregulation. Summary of the mechanisms by which hemolysis, hemoglobin and heme induces oxidative stress, inflammation and thrombosis.

1. Munro AWG, H.M.; McLean, K.J.; Cheesman, M.R.; Leys, D. *Heme and hemoproteins. In tetrapyrroles*. New York, NY, USA: Springer; 2009.
2. Gell DA. Structure and function of haemoglobins. *Blood Cells Mol Dis*. 2018;70:13-42
3. Ascenzi P, Bocedi A, Visca P, Altruda F, Tolosano E, Beringhelli T, Fasano M. Hemoglobin and heme scavenging. *IUBMB Life*. 2005;57:749-759
4. Vallelian F, Buehler PW, Schaer DJ. Hemolysis, free hemoglobin toxicity, and scavenger protein therapeutics. *Blood*. 2022;140:1837-1844
5. Ascenzi P, Fasano M. Serum heme-albumin: An allosteric protein. *IUBMB Life*. 2009;61:1118-1122
6. Dimitrov JD, Roumenina LT, Doltchinkova VR, Mihaylova NM, Lacroix-Desmazes S, Kaveri SV, Vassilev TL. Antibodies use heme as a cofactor to extend their pathogen elimination activity and to acquire new effector functions. *J Biol Chem*. 2007;282:26696-26706
7. Larsson J, Allhorn M, Kerstrom B. The lipocalin alpha(1)-microglobulin binds heme in different species. *Arch Biochem Biophys*. 2004;432:196-204
8. Roumenina LT, Radanova M, Atanasov BP, Popov KT, Kaveri SV, Lacroix-Desmazes S, Fremeaux-Bacchi V, Dimitrov JD. Heme interacts with c1q and inhibits the classical complement pathway. *J Biol Chem*. 2011;286:16459-16469
9. Frimat M, Tabarin F, Dimitrov JD, Poitou C, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, Roumenina LT. Complement activation by heme as a secondary hit for atypical hemolytic uremic syndrome. *Blood*. 2013;122:282-292
10. Repesse Y, Dimitrov JD, Peyron I, Farrokhi Moshai E, Kiger L, Dasgupta S, Delignat S, Marden MC, Kaveri SV, Lacroix-Desmazes S. Heme binds to factor viii and inhibits its interaction with activated factor ix. *J Thromb Haemost*. 2012;10:1062-1071
11. Orino K. Functional binding analysis of human fibrinogen as an iron- and heme-binding protein. *Biometals*. 2013;26:789-794
12. Soares MP, Bach FH. Heme oxygenase-1: From biology to therapeutic potential. *Trends Mol Med*. 2009;15:50-58
13. Medina MV, Sapochnik D, Garcia Sola M, Coso O. Regulation of the expression of heme oxygenase-1: Signal transduction, gene promoter activation, and beyond. *Antioxid Redox Signal*. 2020;32:1033-1044

14. Chau JY, Tiffany CM, Nimishakavi S, Lawrence JA, Pakpour N, Mooney JP, Lokken KL, Caughey GH, Tsolis RM, Luckhart S. Malaria-associated l-arginine deficiency induces mast cell-associated disruption to intestinal barrier defenses against nontyphoidal salmonella bacteremia. *Infect Immun*. 2013;81:3515-3526
15. Kumar S, Bandyopadhyay U. Free heme toxicity and its detoxification systems in human. *Toxicol Lett*. 2005;157:175-188
16. Mancek-Keber M, Frank-Bertoncelj M, Hafner-Bratkovic I, Smole A, Zorko M, Pirher N, Hayer S, Kralj-Iglic V, Rozman B, Ilc N, Horvat S, Jerala R. Toll-like receptor 4 senses oxidative stress mediated by the oxidation of phospholipids in extracellular vesicles. *Sci Signal*. 2015;8:ra60
17. Bekkering S, Quintin J, Joosten LA, van der Meer JW, Netea MG, Riksen NP. Oxidized low-density lipoprotein induces long-term proinflammatory cytokine production and foam cell formation via epigenetic reprogramming of monocytes. *Arterioscler Thromb Vasc Biol*. 2014;34:1731-1738
18. Seong SY, Matzinger P. Hydrophobicity: An ancient damage-associated molecular pattern that initiates innate immune responses. *Nat Rev Immunol*. 2004;4:469-478
19. Soares MP, Bozza MT. Red alert: Labile heme is an alarmin. *Curr Opin Immunol*. 2016;38:94-100
20. Dutra FF, Bozza MT. Heme on innate immunity and inflammation. *Front Pharmacol*. 2014;5:115
21. Pradhan P, Vijayan V, Gueler F, Immenschuh S. Interplay of heme with macrophages in homeostasis and inflammation. *Int J Mol Sci*. 2020;21
22. Martins R, Knapp S. Heme and hemolysis in innate immunity: Adding insult to injury. *Curr Opin Immunol*. 2018;50:14-20
23. Liu Y, Jing F, Yi W, Mendelson A, Shi P, Walsh R, Friedman DF, Minniti C, Manwani D, Chou ST, Yazdanbakhsh K. Ho-1(hi) patrolling monocytes protect against vaso-occlusion in sickle cell disease. *Blood*. 2018;131:1600-1610
24. Singhal R, Rathore DK, Bhakuni T, Seth T, Guchhait P. Absence of nonclassical monocytes in hemolytic patients: Free hb and no-mediated mechanism. *J Immunol Res*. 2019;2019:1409383
25. Allali S, Rignault-Bricard R, de Montalembert M, Taylor M, Bouceba T, Hermine O, Maciel TT. Hbs promotes tlr4-mediated monocyte activation and proinflammatory cytokine production in sickle cell disease. *Blood*. 2022;140:1972-1982
26. Figueiredo RT, Fernandez PL, Mourao-Sa DS, Porto BN, Dutra FF, Alves LS, Oliveira MF, Oliveira PL, Graca-Souza AV, Bozza MT. Characterization of heme as activator of toll-like receptor 4. *J Biol Chem*. 2007;282:20221-20229
27. Fortes GB, Alves LS, de Oliveira R, Dutra FF, Rodrigues D, Fernandez PL, Souto-Padron T, De Rosa MJ, Kelliher M, Golenbock D, Chan FK, Bozza MT. Heme induces programmed necrosis on macrophages through autocrine tnf and ros production. *Blood*. 2012;119:2368-2375
28. Belcher JD, Zhang P, Nguyen J, Kiser ZM, Nath KA, Hu J, Trent JO, Vercellotti GM. Identification of a heme activation site on the md-2/tlr4 complex. *Front Immunol*. 2020;11:1370
29. Janciauskiene S, Vijayan V, Immenschuh S. Tlr4 signaling by heme and the role of heme-binding blood proteins. *Front Immunol*. 2020;11:1964

30. Zhang P, Nguyen J, Abdulla F, Nelson AT, Beckman JD, Vercellotti GM, Belcher JD. Soluble md-2 and heme in sickle cell disease plasma promote pro-inflammatory signaling in endothelial cells. *Front Immunol*. 2021;12:632709
31. Lin J, Lin K, Huang L, Jiang Y, Ding X, Luo W, Samorodov AV, Pavlov VN, Liang G, Qian J, Wang Y. Heme induces inflammatory injury by directly binding to the complex of myeloid differentiation protein 2 and toll-like receptor 4. *Toxicol Lett*. 2022;370:15-23
32. Lin S, Yin Q, Zhong Q, Lv FL, Zhou Y, Li JQ, Wang JZ, Su BY, Yang QW. Heme activates tlr4-mediated inflammatory injury via myd88/trif signaling pathway in intracerebral hemorrhage. *J Neuroinflammation*. 2012;9:46
33. Belcher JD, Chen C, Nguyen J, Milbauer L, Abdulla F, Alayash AI, Smith A, Nath KA, Hebbel RP, Vercellotti GM. Heme triggers tlr4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. *Blood*. 2014;123:377-390
34. Ghosh S, Adisa OA, Chappa P, Tan F, Jackson KA, Archer DR, Ofori-Acquah SF. Extracellular hemin crisis triggers acute chest syndrome in sickle mice. *J Clin Invest*. 2013;123:4809-4820
35. Chen C, Zhang F, Zhang Z, Peng M, Wang Y, Chen Y. Tlr4 signaling-induced heme oxygenase upregulation in the acute lung injury: Role in hemorrhagic shock and two-hit induced lung inflammation. *Mol Biol Rep*. 2013;40:1167-1172
36. Merle NS, Paule R, Leon J, Daugan M, Robe-Rybkine T, Poillerat V, Torset C, Fremeaux-Bacchi V, Dimitrov JD, Roumenina LT. P-selectin drives complement attack on endothelium during intravascular hemolysis in tlr-4/heme-dependent manner. *Proc Natl Acad Sci U S A*. 2019;116:6280-6285
37. Bozza MT, Jeney V. Pro-inflammatory actions of heme and other hemoglobin-derived damps. *Front Immunol*. 2020;11:1323
38. Beckman JD, Abdullah F, Chen C, Kirchner R, Rivera-Rodriguez D, Kiser ZM, Nguyen A, Zhang P, Nguyen J, Hebbel RP, Belcher JD, Vercellotti GM. Endothelial tlr4 expression mediates vaso-occlusive crisis in sickle cell disease. *Front Immunol*. 2020;11:613278
39. Vazquez-Carballo C, Herencia C, Guerrero-Hue M, Garcia-Caballero C, Rayego-Mateos S, Morgado-Pascual JL, Opazo-Rios L, Gonzalez-Guerrero C, Vallejo-Mudarra M, Cortegano I, Gaspar ML, de Andres B, Egido J, Moreno JA. Role of toll-like receptor 4 in intravascular hemolysis-mediated injury. *J Pathol*. 2022;258:236-249
40. Coban C, Ishii KJ, Kawai T, Hemmi H, Sato S, Uematsu S, Yamamoto M, Takeuchi O, Itagaki S, Kumar N, Horii T, Akira S. Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J Exp Med*. 2005;201:19-25
41. Prestes EB, Alves LS, Rodrigues DAS, Dutra FF, Fernandez PL, Paiva CN, Kagan JC, Bozza MT. Mitochondrial reactive oxygen species participate in signaling triggered by heme in macrophages and upon hemolysis. *J Immunol*. 2020;205:2795-2805
42. Bolivar BE, Brown-Suedel AN, Rohrman BA, Charendoff CI, Yazdani V, Belcher JD, Vercellotti GM, Flanagan JM, Bouchier-Hayes L. Noncanonical roles of caspase-4 and caspase-5 in heme-driven il-1beta release and cell death. *J Immunol*. 2021;206:1878-1889
43. Dutra FF, Alves LS, Rodrigues D, Fernandez PL, de Oliveira RB, Golenbock DT, Zamboni DS, Bozza MT. Hemolysis-induced lethality involves inflammasome activation by heme. *Proc Natl Acad Sci U S A*. 2014;111:E4110-4118
44. Haldar M, Kohyama M, So AY, Kc W, Wu X, Briseno CG, Satpathy AT, Kretzer NM, Arase H, Rajasekaran NS, Wang L, Egawa T, Igarashi K, Baltimore D, Murphy TL, Murphy KM.

Heme-mediated spi-c induction promotes monocyte differentiation into iron-recycling macrophages. *Cell*. 2014;156:1223-1234
45. Pfefferle M, Ingoglia G, Schaer CA, Yalamanoglu A, Buzzi R, Dubach IL, Tan G, Lopez-Cano EY, Schulthess N, Hansen K, Humar R, Schaer DJ, Vallelian F. Hemolysis transforms liver macrophages into antiinflammatory erythrophagocytes. *J Clin Invest*. 2020;130:5576-5590
46. Liu Y, Pal M, Bao W, Shi PA, Lobo CA, An X, Manwani D, Zhong H, Yazdanbakhsh K. Type i interferon is induced by hemolysis and drives antibody-mediated erythrophagocytosis in sickle cell disease. *Blood*. 2021;138:1162-1171
47. Dimitrov JD, Roumenina LT. Heme: Driver of erythrocyte elimination. *Blood*. 2021;138:1092-1094
48. McIntyre JA, Faulk WP. Redox-reactive autoantibodies: Biochemistry, characterization, and specificities. *Clin Rev Allergy Immunol*. 2009;37:49-54
49. Roumenina LT, Rayes J, Lacroix-Desmazes S, Dimitrov JD. Heme: Modulator of plasma systems in hemolytic diseases. *Trends Mol Med*. 2016;22:200-213
50. Planchais C, Noe R, Gilbert M, Lecerf M, Kaveri SV, Lacroix-Desmazes S, Roumenina LT, Dimitrov JD. Oxidized hemoglobin triggers polyreactivity and autoreactivity of human igg via transfer of heme. *Commun Biol*. 2023;6:168
51. Martins R, Maier J, Gorki AD, Huber KV, Sharif O, Starkl P, Saluzzo S, Quattrone F, Gawish R, Lakovits K, Aichinger MC, Radic-Sarikas B, Lardeau CH, Hladik A, Korosec A, Brown M, Vaahtomeri K, Duggan M, Kerjaschki D, Esterbauer H, Colinge J, Eisenbarth SC, Decker T, Bennett KL, Kubicek S, Sixt M, Superti-Furga G, Knapp S. Heme drives hemolysis-induced susceptibility to infection via disruption of phagocyte functions. *Nat Immunol*. 2016;17:1361-1372
52. Olonisakin TF, Suber T, Gonzalez-Ferrer S, Xiong Z, Penaloza HF, van der Geest R, Xiong Y, Osei-Hwedieh DO, Tejero J, Rosengart MR, Mars WM, Van Tyne D, Perlegas A, Brashears S, Kim-Shapiro DB, Gladwin MT, Bachman MA, Hod EA, St Croix C, Tyurina YY, Kagan VE, Mallampalli RK, Ray A, Ray P, Lee JS. Stressed erythrophagocytosis induces immunosuppression during sepsis through heme-mediated stat1 dysregulation. *J Clin Invest*. 2021;131
53. May O, Yatime L, Merle NS, Delguste F, Howsam M, Daugan MV, Paul-Constant C, Billamboz M, Ghinet A, Lancel S, Dimitrov JD, Boulanger E, Roumenina LT, Frimat M. The receptor for advanced glycation end products is a sensor for cell-free heme. *FEBS J*. 2021;288:3448-3464
54. May O, Merle NS, Grunenwald A, Gnemmi V, Leon J, Payet C, Robe-Rybkine T, Paule R, Delguste F, Satchell SC, Mathieson PW, Hazzan M, Boulanger E, Dimitrov JD, Fremeaux-Bacchi V, Frimat M, Roumenina LT. Heme drives susceptibility of glomerular endothelium to complement overactivation due to inefficient upregulation of heme oxygenase-1. *Front Immunol*. 2018;9:3008
55. Graca-Souza AV, Arruda MA, de Freitas MS, Barja-Fidalgo C, Oliveira PL. Neutrophil activation by heme: Implications for inflammatory processes. *Blood*. 2002;99:4160-4165
56. Miguel LI, Leonardo FC, Torres LS, Garcia F, Mendonca R, Ferreira WA, Jr., Gotardo EMF, Fabris FCZ, Brito PL, Costa FF, Conran N. Heme induces significant neutrophil adhesion in vitro via an nfkappab and reactive oxygen species-dependent pathway. *Mol Cell Biochem*. 2021;476:3963-3974

57. Arruda MA, Rossi AG, de Freitas MS, Barja-Fidalgo C, Graca-Souza AV. Heme inhibits human neutrophil apoptosis: Involvement of phosphoinositide 3-kinase, mapk, and nf-kappab. *J Immunol*. 2004;173:2023-2030
58. Porto BN, Alves LS, Fernandez PL, Dutra TP, Figueiredo RT, Graca-Souza AV, Bozza MT. Heme induces neutrophil migration and reactive oxygen species generation through signaling pathways characteristic of chemotactic receptors. *J Biol Chem*. 2007;282:24430-24436
59. Evans C, Orf K, Horvath E, Levin M, De La Fuente J, Chakravorty S, Cunnington AJ. Impairment of neutrophil oxidative burst in children with sickle cell disease is associated with heme oxygenase-1. *Haematologica*. 2015;100:1508-1516
60. Chen G, Zhang D, Fuchs TA, Manwani D, Wagner DD, Frenette PS. Heme-induced neutrophil extracellular traps contribute to the pathogenesis of sickle cell disease. *Blood*. 2014;123:3818-3827
61. Kono M, Saigo K, Takagi Y, Takahashi T, Kawauchi S, Wada A, Hashimoto M, Minami Y, Imoto S, Takenokuchi M, Morikawa T, Funakoshi K. Heme-related molecules induce rapid production of neutrophil extracellular traps. *Transfusion*. 2014;54:2811-2819
62. NaveenKumar SK, Hemshekhar M, Sharathbabu BN, Kemparaju K, Mugesh G, Girish KS. Platelet activation and ferroptosis mediated netosis drives heme induced pulmonary thrombosis. *Biochim Biophys Acta Mol Basis Dis*. 2023:166688
63. Merle NS, Noe R, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, Roumenina LT. Complement system part ii: Role in immunity. *Front Immunol*. 2015;6:257
64. Gerogianni A, Dimitrov JD, Zarantonello A, Poillerat V, Chonat S, Sandholm K, McAdam KE, Ekdahl KN, Mollnes TE, Mohlin C, Roumenina LT, Nilsson PH. Heme interferes with complement factor i-dependent regulation by enhancing alternative pathway activation. *Front Immunol*. 2022;13:901876
65. Pawluczkowycz AW, Lindorfer MA, Waitumbi JN, Taylor RP. Hematin promotes complement alternative pathway-mediated deposition of c3 activation fragments on human erythrocytes: Potential implications for the pathogenesis of anemia in malaria. *J Immunol*. 2007;179:5543-5552
66. Lindorfer MA, Cook EM, Reis ES, Ricklin D, Risitano AM, Lambris JD, Taylor RP. Compstatin cp40 blocks hematin-mediated deposition of c3b fragments on erythrocytes: Implications for treatment of malarial anemia. *Clin Immunol*. 2016;171:32-35
67. Frimat M, Boudhabhay I, Roumenina LT. Hemolysis derived products toxicity and endothelium: Model of the second hit. *Toxins (Basel)*. 2019;11
68. Merle NS, Grunenwald A, Rajaratnam H, Gnemmi V, Frimat M, Figueres ML, Knockaert S, Bouzekri S, Charue D, Noe R, Robe-Rybkine T, Le-Hoang M, Brinkman N, Gentinetta T, Edler M, Petrillo S, Tolosano E, Miescher S, Le Jeune S, Houillier P, Chauvet S, Rabant M, Dimitrov JD, Fremeaux-Bacchi V, Blanc-Brude OP, Roumenina LT. Intravascular hemolysis activates complement via cell-free heme and heme-loaded microvesicles. *JCI Insight*. 2018;3
69. Merle NS, Leon J, Poillerat V, Grunenwald A, Boudhabhay I, Knockaert S, Robe-Rybkine T, Torset C, Pickering MC, Chauvet S, Fremeaux-Bacchi V, Roumenina LT. Circulating fh protects kidneys from tubular injury during systemic hemolysis. *Front Immunol*. 2020;11:1772
70. Roumenina LT, Bartolucci P, Pirenne F. The role of complement in post-transfusion hemolysis and hyperhemolysis reaction. *Transfus Med Rev*. 2019;33:225-230

71. Boudhabhay I, Poillerat V, Grunenwald A, Torset C, Leon J, Daugan MV, Lucibello F, El Karoui K, Ydee A, Chauvet S, Girardie P, Sacks S, Farrar CA, Garred P, Berthaud R, Le Quintrec M, Rabant M, de Lonlay P, Rambaud C, Gnemmi V, Fremeaux-Bacchi V, Frimat M, Roumenina LT. Complement activation is a crucial driver of acute kidney injury in rhabdomyolysis. *Kidney Int*. 2021;99:581-597
72. Dimitrov JD, Roumenina LT, Doltchinkova VR, Vassilev TL. Iron ions and haeme modulate the binding properties of complement subcomponent c1q and of immunoglobulins. *Scand J Immunol*. 2007;65:230-239
73. Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon RO, 3rd, Schechter AN, Gladwin MT. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat Med*. 2002;8:1383-1389
74. Camus SM, Gausseres B, Bonnin P, Loufrani L, Grimaud L, Charue D, De Moraes JA, Renard JM, Tedgui A, Boulanger CM, Tharaux PL, Blanc-Brude OP. Erythrocyte microparticles can induce kidney vaso-occlusions in a murine model of sickle cell disease. *Blood*. 2012;120:5050-5058
75. Fens MH, van Wijk R, Andringa G, van Rooijen KL, Dijstelbloem HM, Rasmussen JT, de Vooght KM, Schiffelers RM, Gaillard CA, van Solinge WW. A role for activated endothelial cells in red blood cell clearance: Implications for vasopathology. *Haematologica*. 2012;97:500-508
76. Grutzendler J, Murikinati S, Hiner B, Ji L, Lam CK, Yoo T, Gupta S, Hafler BP, Adelman RA, Yuan P, Rodriguez G. Angiophagy prevents early embolus washout but recanalizes microvessels through embolus extravasation. *Sci Transl Med*. 2014;6:226ra231
77. Chang R, Castillo J, Zambon AC, Krasieva TB, Fisher MJ, Sumbria RK. Brain endothelial erythrophagocytosis and hemoglobin transmigration across brain endothelium: Implications for pathogenesis of cerebral microbleeds. *Front Cell Neurosci*. 2018;12:279
78. Roth RI. Hemoglobin enhances the production of tissue factor by endothelial cells in response to bacterial endotoxin. *Blood*. 1994;83:2860-2865
79. Wagener FA, Feldman E, de Witte T, Abraham NG. Heme induces the expression of adhesion molecules icam-1, vcam-1, and e selectin in vascular endothelial cells. *Proc Soc Exp Biol Med*. 1997;216:456-463
80. Lisk C, Kominsky D, Ehrentraut S, Bonaventura J, Nuss R, Hassell K, Nozik-Grayck E, Irwin DC. Hemoglobin-induced endothelial cell permeability is controlled, in part, via a myeloid differentiation primary response gene-88-dependent signaling mechanism. *Am J Respir Cell Mol Biol*. 2013;49:619-626
81. Sparkenbaugh EM, Chantrathammachart P, Wang S, Jonas W, Kirchhofer D, Gailani D, Gruber A, Kasthuri R, Key NS, Mackman N, Pawlinski R. Excess of heme induces tissue factor-dependent activation of coagulation in mice. *Haematologica*. 2015;100:308-314
82. Solovey A, Kollander R, Shet A, Milbauer LC, Choong S, Panoskaltsis-Mortari A, Blazar BR, Kelm RJ, Jr., Hebbel RP. Endothelial cell expression of tissue factor in sickle mice is augmented by hypoxia/reoxygenation and inhibited by lovastatin. *Blood*. 2004;104:840-846
83. Ataga KI, Kutlar A, Kanter J, Liles D, Cancado R, Friedrisch J, Guthrie TH, Knight-Madden J, Alvarez OA, Gordeuk VR, Gualandro S, Colella MP, Smith WR, Rollins SA, Stocker JW, Rother RP. Crizanlizumab for the prevention of pain crises in sickle cell disease. *N Engl J Med*. 2017;376:429-439

84. Vats R, Kaminski TW, Ju EM, Brozska T, Tutuncuoglu E, Tejero J, Novelli EM, Sundd P, Pradhan-Sundd T. P-selectin deficiency promotes liver senescence in sickle cell disease mice. *Blood*. 2021;137:2676-2680
85. Erdei J, Toth A, Balogh E, Nyakundi BB, Banyai E, Ryffel B, Paragh G, Cordero MD, Jeney V. Induction of nlrp3 inflammasome activation by heme in human endothelial cells. *Oxid Med Cell Longev*. 2018;2018:4310816
86. Higdon AN, Benavides GA, Chacko BK, Ouyang X, Johnson MS, Landar A, Zhang J, Darley-Usmar VM. Hemin causes mitochondrial dysfunction in endothelial cells through promoting lipid peroxidation: The protective role of autophagy. *Am J Physiol Heart Circ Physiol*. 2012;302:H1394-1409
87. Camus SM, De Moraes JA, Bonnin P, Abbyad P, Le Jeune S, Lionnet F, Loufrani L, Grimaud L, Lambry JC, Charue D, Kiger L, Renard JM, Larroque C, Le Clesiau H, Tedgui A, Bruneval P, Barja-Fidalgo C, Alexandrou A, Tharaux PL, Boulanger CM, Blanc-Brude OP. Circulating cell membrane microparticles transfer heme to endothelial cells and trigger vasoocclusions in sickle cell disease. *Blood*. 2015;125:3805-3814
88. Singla S, Sysol JR, Dille B, Jones N, Chen J, Machado RF. Hemin causes lung microvascular endothelial barrier dysfunction by necroptotic cell death. *Am J Respir Cell Mol Biol*. 2017;57:307-314
89. Feng S, Liang X, Cruz MA, Vu H, Zhou Z, Pemmaraju N, Dong JF, Kroll MH, Afshar-Kharghan V. The interaction between factor h and von willebrand factor. *PLoS One*. 2013;8:e73715
90. Feng S, Liang X, Kroll MH, Chung DW, Afshar-Kharghan V. Von willebrand factor is a cofactor in complement regulation. *Blood*. 2015;125:1034-1037
91. Noone DG, Riedl M, Pluthero FG, Bowman ML, Liszewski MK, Lu L, Quan Y, Balgobin S, Schneppenheim R, Schneppenheim S, Budde U, James P, Atkinson JP, Palaniyar N, Kahr WH, Licht C. Von willebrand factor regulates complement on endothelial cells. *Kidney Int*. 2016;90:123-134
92. Gremmel T, Fedrizzi S, Weigel G, Eichelberger B, Panzer S. Underlying mechanism and specific prevention of hemolysis-induced platelet activation. *Platelets*. 2017;28:555-559
93. Polanowska-Grabowska R, Wallace K, Field JJ, Chen L, Marshall MA, Figler R, Gear AR, Linden J. P-selectin-mediated platelet-neutrophil aggregate formation activates neutrophils in mouse and human sickle cell disease. *Arterioscler Thromb Vasc Biol*. 2010;30:2392-2399
94. Brzoska T, Vats R, Bennewitz MF, Tutuncuoglu E, Watkins SC, Ragni MV, Neal MD, Gladwin MT, Sundd P. Intravascular hemolysis triggers adp-mediated generation of platelet-rich thrombi in precapillary pulmonary arterioles. *JCI Insight*. 2020;5
95. Heeney MM, Hoppe CC, Abboud MR, Inusa B, Kanter J, Ogutu B, Brown PB, Heath LE, Jakubowski JA, Zhou C, Zamoryakhin D, Agbenyega T, Colombatti R, Hassab HM, Nduba VN, Oyieko JN, Robitaille N, Segbefia CI, Rees DC, Investigators D. A multinational trial of prasugrel for sickle cell vaso-occlusive events. *N Engl J Med*. 2016;374:625-635
96. Bourne JH, Colicchia M, Di Y, Martin E, Slater A, Roumenina LT, Dimitrov JD, Watson SP, Rayes J. Heme induces human and mouse platelet activation through c-type-lectin-like receptor-2. *Haematologica*. 2021;106:626-629

97. Annarapu GK, Nolfi-Donegan D, Reynolds M, Wang Y, Kohut L, Zuckerbraun B, Shiva S. Heme stimulates platelet mitochondrial oxidant production to induce targeted granule secretion. *Redox Biol*. 2021;48:102205
98. NaveenKumar SK, Hemshekhar M, Kemparaju K, Girish KS. Hemin-induced platelet activation and ferroptosis is mediated through ros-driven proteasomal activity and inflammasome activation: Protection by melatonin. *Biochim Biophys Acta Mol Basis Dis*. 2019;1865:2303-2316
99. Singhal R, Annarapu GK, Pandey A, Chawla S, Ojha A, Gupta A, Cruz MA, Seth T, Guchhait P. Hemoglobin interaction with gp1balpha induces platelet activation and apoptosis: A novel mechanism associated with intravascular hemolysis. *Haematologica*. 2015;100:1526-1533
100. Annarapu GK, Singhal R, Peng Y, Guchhait P. Inhibition of hb binding to gp1balpha abrogates hb-mediated thrombus formation on immobilized vwf and collagen under physiological shear stress. *PLoS One*. 2016;11:e0154276
101. Zhou Z, Han H, Cruz MA, Lopez JA, Dong JF, Guchhait P. Haemoglobin blocks von willebrand factor proteolysis by adamts-13: A mechanism associated with sickle cell disease. *Thromb Haemost*. 2009;101:1070-1077
102. Asher C, de Villiers KA, Egan TJ. Speciation of ferriprotoporphyrin ix in aqueous and mixed aqueous solution is controlled by solvent identity, ph, and salt concentration. *Inorg Chem*. 2009;48:7994-8003
103. Hopp MT, Imhof D. Linking labile heme with thrombosis. *J Clin Med*. 2021;10
104. Hopp MT, Alhanafi N, Paul George AA, Hamedani NS, Biswas A, Oldenburg J, Potzsch B, Imhof D. Molecular insights and functional consequences of the interaction of heme with activated protein c. *Antioxid Redox Signal*. 2021;34:32-48
105. Moore HB, Moore EE, Gonzalez E, Hansen KC, Dzieciatkowska M, Chapman MP, Sauaia A, West B, Banerjee A, Silliman CC. Hemolysis exacerbates hyperfibrinolysis, whereas platelolysis shuts down fibrinolysis: Evolving concepts of the spectrum of fibrinolysis in response to severe injury. *Shock*. 2015;43:39-46
106. Barrera V, Skorokhod OA, Baci D, Gremo G, Arese P, Schwarzer E. Host fibrinogen stably bound to hemozoin rapidly activates monocytes via tlr-4 and cd11b/cd18-integrin: A new paradigm of hemozoin action. *Blood*. 2011;117:5674-5682
107. NaveenKumar SK, SharathBabu BN, Hemshekhar M, Kemparaju K, Girish KS, Mugesh G. The role of reactive oxygen species and ferroptosis in heme-mediated activation of human platelets. *ACS Chem Biol*. 2018;13:1996-2002
108. Oishi S, Tsukiji N, Otake S, Oishi N, Sasaki T, Shirai T, Yoshikawa Y, Takano K, Shinmori H, Inukai T, Kondo T, Suzuki-Inoue K. Heme activates platelets and exacerbates rhabdomyolysis-induced acute kidney injury via clec-2 and gpvi/fcrgamma. *Blood Adv*. 2021;5:2017-2026
109. Rayes J, Watson SP, Nieswandt B. Functional significance of the platelet immune receptors gpvi and clec-2. *J Clin Invest*. 2019;129:12-23
110. Okubo K, Kurosawa M, Kamiya M, Urano Y, Suzuki A, Yamamoto K, Hase K, Homma K, Sasaki J, Miyauchi H, Hoshino T, Hayashi M, Mayadas TN, Hirahashi J. Macrophage extracellular trap formation promoted by platelet activation is a key mediator of rhabdomyolysis-induced acute kidney injury. *Nat Med*. 2018;24:232-238
111. Peng L, Mundada L, Stomel JM, Liu JJ, Sun J, Yet SF, Fay WP. Induction of heme oxygenase-1 expression inhibits platelet-dependent thrombosis. *Antioxid Redox Signal*. 2004;6:729-735

112. Cubedo J, Suades R, Padro T, Martin-Yuste V, Sabate-Tenas M, Cinca J, Sans-Rosello J, Sionis A, Badimon L. Erythrocyte-heme proteins and stemi: Implications in prognosis. *Thromb Haemost*. 2017;117:1970-1980
113. Stolla M, Henrichs K, Cholette JM, Pietropaoli AP, Phipps RP, Spinelli SL, Blumberg N. Haem is associated with thrombosis in neonates and infants undergoing cardiac surgery for congenital heart disease. *Vox Sang*. 2018;113:72-75
114. True AL, Olive M, Boehm M, San H, Westrick RJ, Raghavachari N, Xu X, Lynn EG, Sack MN, Munson PJ, Gladwin MT, Nabel EG. Heme oxygenase-1 deficiency accelerates formation of arterial thrombosis through oxidative damage to the endothelium, which is rescued by inhaled carbon monoxide. *Circ Res*. 2007;101:893-901
115. Woollard KJ, Sturgeon S, Chin-Dusting JP, Salem HH, Jackson SP. Erythrocyte hemolysis and hemoglobin oxidation promote ferric chloride-induced vascular injury. *J Biol Chem*. 2009;284:13110-13118
116. Nath KA, Grande JP, Belcher JD, Garovic VD, Croatt AJ, Hillestad ML, Barry MA, Nath MC, Regan RF, Vercellotti GM. Antithrombotic effects of heme-degrading and heme-binding proteins. *Am J Physiol Heart Circ Physiol*. 2020;318:H671-H681
117. Payne H, Ponomaryov T, Watson SP, Brill A. Mice with a deficiency in clec-2 are protected against deep vein thrombosis. *Blood*. 2017;129:2013-2020
118. Reutershan J, Harry B, Chang D, Bagby GJ, Ley K. Darc on rbc limits lung injury by balancing compartmental distribution of cxc chemokines. *Eur J Immunol*. 2009;39:1597-1607
119. Afenyi-Annan A, Kail M, Combs MR, Orringer EP, Ashley-Koch A, Telen MJ. Lack of duffy antigen expression is associated with organ damage in patients with sickle cell disease. *Transfusion*. 2008;48:917-924
120. Tournamille C, Colin Y, Cartron JP, Le Van Kim C. Disruption of a gata motif in the duffy gene promoter abolishes erythroid gene expression in duffy-negative individuals. *Nat Genet*. 1995;10:224-228
121. Pruenster M, Rot A. Throwing light on darc. *Biochem Soc Trans*. 2006;34:1005-1008
122. Darbonne WC, Rice GC, Mohler MA, Apple T, Hebert CA, Valente AJ, Baker JB. Red blood cells are a sink for interleukin 8, a leukocyte chemotaxin. *J Clin Invest*. 1991;88:1362-1369
123. Hotz MJ, Qing D, Shashaty MGS, Zhang P, Faust H, Sondheimer N, Rivella S, Worthen GS, Mangalmurti NS. Red blood cells homeostatically bind mitochondrial DNA through tlr9 to maintain quiescence and to prevent lung injury. *Am J Respir Crit Care Med*. 2018;197:470-480
124. Lam LKM, Murphy S, Kokkinaki D, Venosa A, Sherrill-Mix S, Casu C, Rivella S, Weiner A, Park J, Shin S, Vaughan AE, Hahn BH, Odom John AR, Meyer NJ, Hunter CA, Worthen GS, Mangalmurti NS. DNA binding to tlr9 expressed by red blood cells promotes innate immune activation and anemia. *Sci Transl Med*. 2021;13:eabj1008
125. Donegan RK. The role of host heme in bacterial infection. *Biol Chem*. 2022;403:1017-1029
126. Larsen R, Gozzelino R, Jeney V, Tokaji L, Bozza FA, Japiassu AM, Bonaparte D, Cavalcante MM, Chora A, Ferreira A, Marguti I, Cardoso S, Sepulveda N, Smith A, Soares MP. A central role for free heme in the pathogenesis of severe sepsis. *Sci Transl Med*. 2010;2:51ra71

127. Ochocinski D, Dalal M, Black LV, Carr S, Lew J, Sullivan K, Kissoon N. Life-threatening infectious complications in sickle cell disease: A concise narrative review. *Front Pediatr*. 2020;8:38
128. Cunnington AJ, de Souza JB, Walther M, Riley EM. Malaria impairs resistance to salmonella through heme- and heme oxygenase-dependent dysfunctional granulocyte mobilization. *Nat Med*. 2011;18:120-127
129. Zhong H, Bao W, Friedman D, Yazdanbakhsh K. Hemin controls t cell polarization in sickle cell alloimmunization. *J Immunol*. 2014;193:102-110
130. Watanabe-Matsui M, Muto A, Matsui T, Itoh-Nakadai A, Nakajima O, Murayama K, Yamamoto M, Ikeda-Saito M, Igarashi K. Heme regulates b-cell differentiation, antibody class switch, and heme oxygenase-1 expression in b cells as a ligand of bach2. *Blood*. 2011;117:5438-5448
131. Janz DR, Bastarache JA, Peterson JF, Sills G, Wickersham N, May AK, Roberts LJ, 2nd, Ware LB. Association between cell-free hemoglobin, acetaminophen, and mortality in patients with sepsis: An observational study. *Crit Care Med*. 2013;41:784-790
132. Adamzik M, Hamburger T, Petrat F, Peters J, de Groot H, Hartmann M. Free hemoglobin concentration in severe sepsis: Methods of measurement and prediction of outcome. *Crit Care*. 2012;16:R125

Figure 1

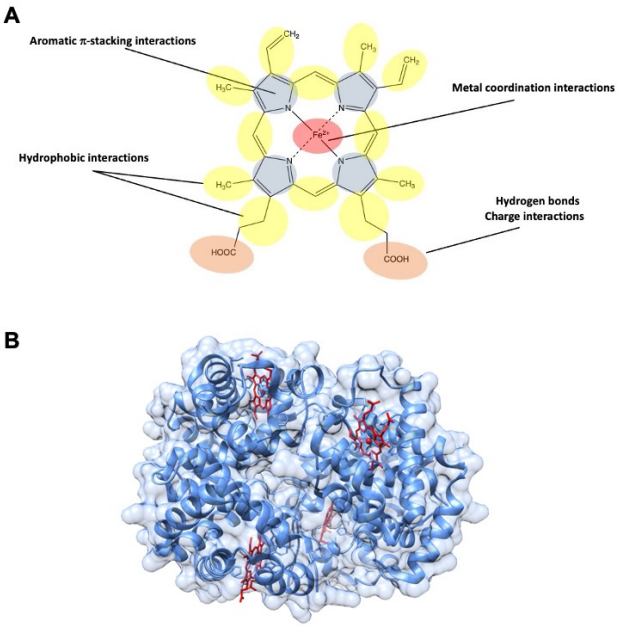
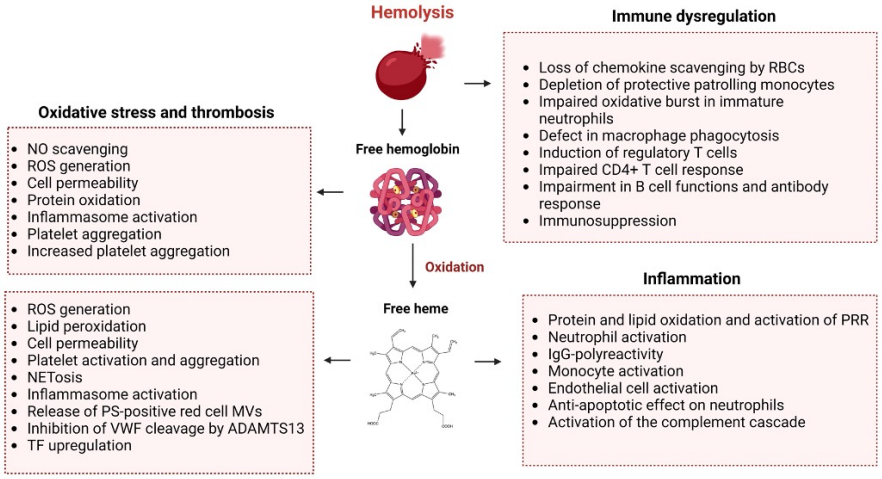

A
Aromatic π-stacking interactions
Metal coordination interactions
Hydrophobic interactions
Hydrogen bonds
Charge interactions
HOOC
COOH
B

Figure 2


Hemolysis
Immune dysregulation
Loss of chemokine scavenging by RBCs
Depletion of protective patrolling monocytes
Impaired oxidative burst in immature neutrophils
Defect in macrophage phagocytosis
Induction of regulatory T cells
Impaired CD4+ T cell response
Impairment in B cell functions and antibody response
Immunosuppression
Oxidative stress and thrombosis
NO scavenging
ROS generation
Cell permeability
Protein oxidation
Inflammasome activation
Platelet aggregation
Increased platelet aggregation
Free hemoglobin
Oxidation
Free heme
ROS generation
Lipid peroxidation
Cell permeability
Platelet activation and aggregation
NETosis
Inflammasome activation
Release of PS-positive red cell MVs
Inhibition of VWF cleavage by ADAMTS13
TF upregulation
Inflammation
Protein and lipid oxidation and activation of PRR
Neutrophil activation
IgG-polyreactivity
Monocyte activation
Endothelial cell activation
Anti-apoptotic effect on neutrophils
Activation of the complement cascade