Investigation of the procoagulant role of labile heme under hemolytic conditions

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"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.[1]*"*

~ Marie Skłodowska-Curie ~
(1867-1934)

Abstract

Severe intravascular hemolysis leads to the excessive release of labile heme into the vascular compartment, thereby overwhelming the heme-binding capacity of the protective heme-scavenging system. As a consequence, labile heme activates proinflammatory signaling pathways and the complement system, which is often regulated by transient heme binding to involved proteins. Moreover, patients with hemolytic disorders frequently suffer from thrombotic complications. Though these procoagulant responses might be of multifunctional origin, several studies suggest a major role of labile heme. In the present thesis, the current knowledge of heme as a prothrombotic alarmin is comprehensively compiled. This analysis revealed essential knowledge gaps on the level of heme-induced signaling pathways concerning the cellular components of the blood coagulation system, yet also on the level of direct heme-protein interactions that are only rarely investigated so far. Two approaches should be applied herein in order to address both aspects using specific examples. Initially, the binding of heme to the blood coagulation inhibitor activated protein C was investigated. Two heme-regulatory motifs were identified and characterized. The functional consequences of this interaction were analyzed by various biochemical and physiological relevant assays, in particular confirming the procoagulant role of heme by inhibition of the enzyme. In the second approach, data from 46 publications on the effect of heme under hemolytic conditions were curated and contextualized resulting in a network (HemeKG) that allows for detailed analysis of relevant signaling pathways, as exemplified by the TLR4 signaling pathway.

The present thesis contributes to the current knowledge on heme as a prothrombotic molecule through the characterization of the enzyme activated protein C as a further, heme-regulated protein of the blood coagulation system and by providing HemeKG as a basis for future heme-triggered pathway and pathology analysis as well as for the development of suitable drugs for the treatment of hemolysis-driven thrombosis.

Zusammenfassung

Schwere intravaskuläre Hämolyse führt zu einer exzessiven Freisetzung von labilem Häm in das Blutsystem, wodurch die Häm-Bindungskapazität der schützenden Häm-Scavenger-Proteine schnell erschöpft sein kann. Infolgedessen aktiviert labiles Häm proinflammatorische Signalwege und das Komplementsystem, was beispielsweise durch eine transiente Bindung von Häm an beteiligte Proteine ausgelöst wird. Darüber hinaus leiden Patienten mit hämolytischen Krankheiten häufig unter thrombotischen Komplikationen. Obwohl diese gerinnungsfördernden Reaktionen vermutlich durch verschiedene Faktoren beeinflusst werden, deuten verschiedene Studien auf eine tragende Rolle des labilen Häms hin. Im Rahmen der vorliegenden Arbeit wird der aktuelle Wissensstand über die Wirkung von Häm innerhalb der Blutgerinnung umfassend zusammengestellt. Dabei werden wesentliche Wissenslücken auf der Ebene der Häm-induzierten Signalwege innerhalb der zellulären Komponenten des Blutgerinnungssystems sowie auf der Ebene direkter Häm-Protein-Interaktionen aufgezeigt, die bisher nur wenig untersucht sind. Mithilfe zwei verschiedener Ansätze sollten beide Aspekte adressiert werden. Zunächst wurde die Bindung von Häm an den Blutgerinnungsinhibitor aktiviertes Protein C nachgewiesen. Zwei Häm-regulatorische Motive wurden identifiziert und charakterisiert. Die funktionellen Konsequenzen dieser Interaktion wurden mit verschiedenen biochemischen und physiologisch relevanten Tests analysiert, wobei durch die Inhibierung des Enzyms insbesondere die gerinnungsfördernde Rolle von Häm bestätigt wurde. In einem zweiten Ansatz wurden Daten aus 46 Publikationen zur Wirkung von Häm unter hämolytischen Bedingungen herausgearbeitet und in einen Kontext gebracht, was zur Generierung eines Netzwerkes (HemeKG) führte, welches nun die detaillierte Analyse relevanter Signalwege ermöglicht, wie am Beispiel des TLR4-Signalwegs gezeigt wurde.

Die vorliegende Arbeit trägt zum aktuellen Wissensstand über labiles Häm als prothrombotisch wirkendes Molekül bei, indem sie das Enzym aktiviertes Protein C als weiteres, Häm-reguliertes Protein innerhalb des Blutgerinnungssystems charakterisiert und HemeKG als Grundlage für zukünftige Analysen von Häm-induzierten Signalwegen und Krankheiten sowie für die Entwicklung geeigneter Medikamente zur Behandlung von durch Hämolyse verursachten Thrombosen bereitstellt.

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1 Introduction

An adult human takes 18 to 20 breaths per minute, which supplies cells, tissues and organs with oxygen throughout the whole body.[2] The distribution of oxygen is managed by up to ~25 trillion red blood cells (RBCs) that circulate within the blood.[3] Thereby, the porphyrin heme, which is the prosthetic group of the most abundant protein in RBCs, i.e. hemoglobin, is responsible for oxygen binding. Each RBC contains approximately one billion heme molecules, accounting for a total amount of ~25 trillion heme molecules in the human body.[4,5] Under hemolytic conditions, such as in sickle cell disease (SCD), β-thalassemia or paroxysmal nocturnal hemoglobinuria, premature RBC destruction can lead to the release and the accumulation of large amounts of labile heme into the blood stream.[6] As a consequence, heme can induce various harmful effects, leading for example to oxidative stress and the activation of proinflammatory pathways.[6–11] Moreover, patients in acute phases of hemolysis often suffer from thrombosis.[12–17] Since hemolysis is inevitably associated with a massive release of labile heme, the metalloporphyrin has been considered as a critical factor for hypercoagulability in hemolysis. For example, the activation of platelets or direct binding to fibrinogen has been demonstrated in the past.[10,18–21] So far, the prothrombotic role of heme is rather neglected, and the interactions of heme with components of the blood coagulation system and the interrelations thereof are largely unknown. Thus, the present thesis aims at shedding light on the role of labile heme in the context of hemolysis-driven thrombosis. A detailed summary of the knowledge of approximately 110 years of research shall serve as a unique overview of already known interactions of heme with the blood coagulation system on the cellular and the molecular level as a starting point for further investigations. Hence, the focus of a specific study of this work is the complete characterization of heme binding to a potentially heme-regulated protein, i.e. the anticoagulant serine protease activated protein C. Functional analysis in the presence of heme should demonstrate the impact on the anticoagulant activity of the enzyme. Furthermore, data curation of experimentally gained information on the numerous actions of heme shall allow for the crosstalk analysis of heme-triggered effects under hemolytic conditions. The scope of its application should be demonstrated not only by the example of a selected pathway analysis, but also by the investigation of intersections between heme-driven pathology in hemolysis and the coronavirus disease of 2019 (COVID-19) pathogenesis.

2 Theoretical Background

Whole blood accounts for approximately 6-8% of the human body weight. It comprises plasma (~55%) and cellular components (~45%),[22–25] thereby maintaining essential functions, such as oxygen transport or contribution to vital processes, such as blood coagulation and inflammation.[26–28] Among platelets (~2-3 x 10^{11} platelets/l whole blood)[29–31] and white blood cells (WBCs; ~5 x 10^{9} WBCs/l whole blood)[32], red blood cells (RBCs) constitute the largest portion of the total cell count with about 4-5 x 10^{12} RBCs/l whole blood (~25 trillion in circulation; Figure 1),[3,31,33] which roughly corresponds to 35-45% of the total blood volume (*hematocrit*).[34] As a result of hematopoiesis that mainly occurs in the red bone marrow,[35] mature RBCs have a small, biconcave form with a lack of a nucleus and other organelles, such as mitochondria and ribosomes, and, therefore, are not capable of own protein biosynthesis or mitosis.[36] In contrast, RBC possess the ability to synthesize adenosine triphosphate (ATP) and glutathione.[37,38] All of these characteristics ensure the smooth course of the main function of RBCs, the efficient oxygen transport from the lungs to various tissues. The major actor for the delivery of oxygen is the protein hemoglobin. This heterotetramer (~64 kDa) consists of two α-globin and two β-globin chains, each of which contains one heme *b* (in the following referred to as *heme*) molecule permanently bound as a prosthetic group (dissociation constant (K_D) ~182 nM; Figure 1).[39,40] In addition, there is also evidence for the presence of a pool of unbound heme in RBCs (~0.1-21 μM).[4,41] Heme (iron (II) protoporphyrin IX) itself is a complex with the macrocyclic ligand protoporphyrin IX coordinated to a central ferrous iron (II) ion (Figure 1). Four of six coordination sites of the iron (II) ion are occupied by the porphyrin ring, while the fifth coordination site is attached to the proximal histidine residue, His 87 (α-chains) or His 92 (β-chains), of the respective hemoglobin subunit.[39,42,43] Another site is available for the coordination of molecular oxygen, thereby conferring hemoglobin its properties. In order to allow for efficient hemoglobin-driven oxygen transport, the hemoglobin concentration inside RBCs is extremely high (0.25 billion molecules per RBC, ~1.35 g/l whole blood).[3]. Accordingly, since each hemoglobin molecule is capable of binding four O_2 molecules, each RBC can reversibly pick up approximately one billion O_2 molecules.

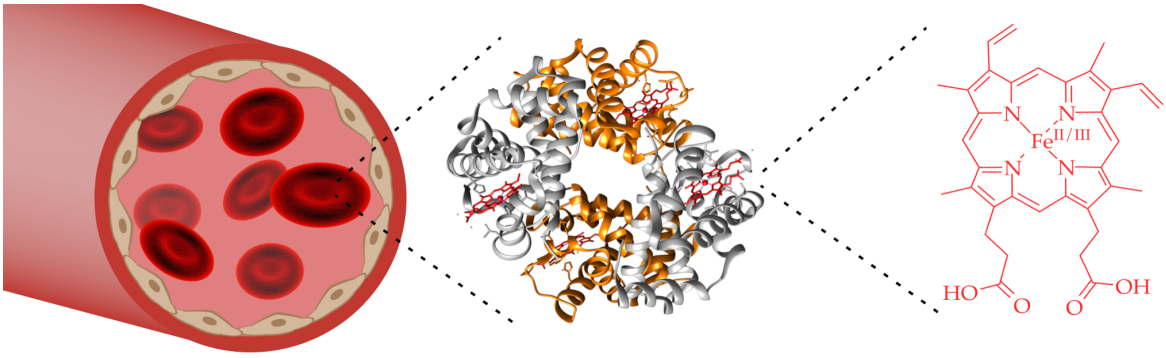

Figure 1: The most common cells in human blood, RBCs, contain hemoglobin. Blood is transported through the body by blood vessels (left). Within the blood there are approximately 2.5 x 10^{13} RBCs circulating, each containing ~2.8 x 10^{8} hemoglobin (middle) molecules as the oxygen-transporting moiety.[3] It consists of two α-chains (orange) and two β-chains (grey). Deoxyhemoglobin is depicted (PDB: 1KD2). Each of its subunits contains a permanently bound heme molecule (red; right) as a prosthetic group. Thus, in each RBC there are approximately one billion heme molecules, which corresponds to a total amount of ~2.5 x 10^{22} heme molecules in the human body.[4,5]

2.1 Physiological processing of senescent red blood cells

RBCs undergo aging processes. Hence, there is not only the need of erythropoiesis but of a controlled RBC degradation as well. Basically, two mechanisms for the controlled degradation of senescent RBCs have been suggested: Eryptosis and Band 3 clustering.[44] Eryptosis, the process of suicidal death of RBCs, can be induced by several stress factors, such as oxidative stress.[45] Consequent decay in enzymes leads to a decline of metabolism and, consequently, to decreased ATP and 2,3-diphosphoglycerate (2,3-DPG) levels.[46,47] While ATP loss results in functional impairment of the K^+/Na^+ ATPase, and, thus, to a change of the essential biconcave to a rigid form of RBCs, lower 2,3-DPG concentrations abolish efficient transfer of oxygen to peripheral tissues.[48,49] Moreover, eryptosis is accompanied by cell blebbing with the formation of hemoglobin-loaded microvesicles (MVs) and exposure of phosphatidylserine.[50,51] The shape deformation of RBCs might restrict unhindered passage through splenic microcirculation due to the loss of flexibility.[52] As a consequence, RBCs are cleared from circulation through phagocytosis by macrophages of the reticuloendothelial system (RES; in particular in the red pulp macrophages of the spleen).[53] In senescent RBCs, autoxidation of hemoglobin (from Fe(II) to Fe(III) in heme) leads to the generation of methemoglobin and reactive oxygen species (ROS).[54] Methemoglobin, which is unable to transport oxygen, is thought to attach in form of low-

spin methemoglobin (hemichrome) to the inner plasma membrane site of RBCs, resulting in the clustering of transmembrane protein Band 3.[55] Subsequently, immunoglobulin G (IgG) is able to bind to Band 3, thereby labeling aged and functional impaired RBCs.[55,56] In addition, phosphatidylserine exposure, adhesion protein activation and reduced superficial cluster of differentiation 47 (CD47) expression has been positively correlated with RBC clearance through interaction with prophagocytic receptors on the surface of macrophages.[57–59] Thus, senescent RBCs are explicitly recognized and engulfed by (mainly) red pulp macrophages.[53] In this way, senescent RBCs are usually removed from circulation after ~120 days, resulting in a turnover of ~360 billion RBCs per day.[60,61]

RBC clearance is inevitably associated with iron recycling.[62,63] Upon erythrophagocytosis, RBC components become proteolytically processed and degraded within the phagolysosome. The RBC content is released into the macrophage cytosol and subsequently either stored or recycled. As such, hemoglobin is readily disrupted, thereby liberating its prosthetic group. Heme, in turn, was shown to be delivered into the macrophage cytosol via the heme responsive gene 1 protein (HRG-1).[64] Subsequent to cytosolic arrival, heme is degraded, catalyzed by peripheral membrane-associated enzymes of the smooth endoplasmatic reticulum (ER), namely heme oxygenases (HOs).[65–67] There are two isoforms, the inducible HO-1 and the constitutive HO-2. While HO-1 is anchored to the ER membrane within macrophages mainly in the spleen and liver, HO-2 is predominantly found in neurons of the brain.[68,69] Due to its constitutive nature, HO-2 is not sensitive to environmental stress, like hemolysis. In contrast, HO-1 expression is usually suppressed by the transcription factor Bach1. High heme levels inhibit Bach1, which leads to the expression of HO-1.[70] Thus, HO-1 is the predominant isoform for the degradation of heme. Ferric heme binds to a hydrophobic pocket out of two α-helices and is coordinated via His 25 of the proximal α-helix (K_D ~350 nM; Figure 2a).[71–74] Upon binding, heme is sequestered through gradual oxidative degradation, which requires three oxygen and three nicotinamide adenine dinucleotide phosphate (NADPH) molecules in total (Figure 2b, c). First, ferric heme is converted into its ferrous state in a NADPH-cytochrome P450 reductase (CPR)-dependent fashion, guaranteeing optimal electron transfer.[74,75] Subsequent binding of oxygen results in the formation of α-*meso*-hydroxyheme (Figure 2c).[76] In the next step, α-verdoheme (Figure 2c) is generated along with an release of carbon monoxide (CO).[77] Rate-limiting ring opening within α-verdoheme and loss of the central iron ion determines the direct conversion into the green pigment biliverdin (Figure 2c).[78,79] By means of the electron donator NADPH and catalyzed by the enzyme biliverdin reductase (BVR), biliverdin is reduced to the yellow-colored unconjugated bilirubin (Figure 2c).[78] Subsequently, unconjugated bilirubin binds to human serum albumin (HSA; 2:1 (bilirubin:HSA); K_{D1} ~0.03 μM, K_{D2} ~2 μM)[80,81] and is transferred to the liver through the blood stream, where it is taken up by hepatocytes via transporters, such as the organic anion-transporting poly-

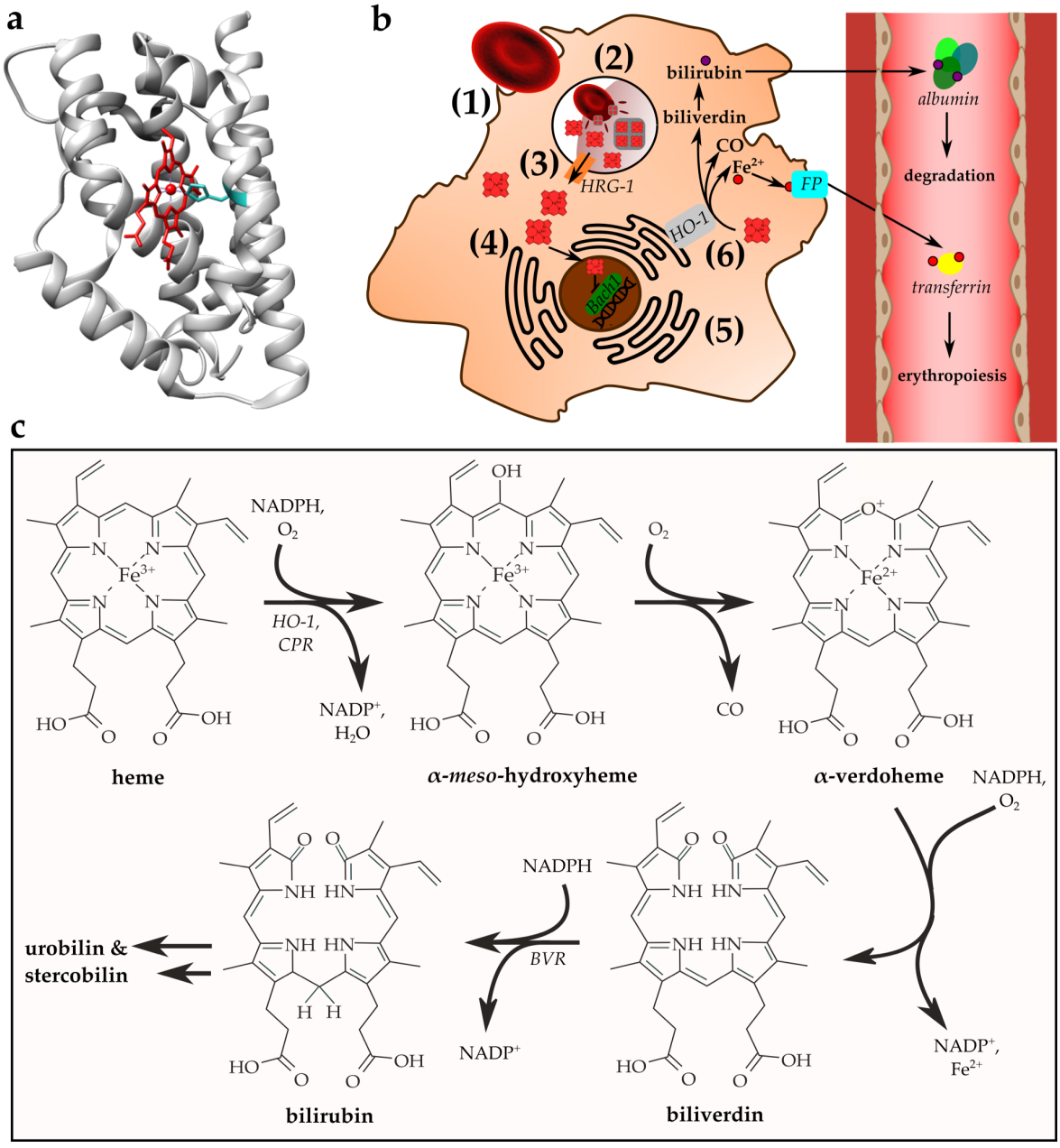

Figure 2: Heme degradation as a consequence of RBC breakdown. a) HO-1 (PDB: 1N3U) binds heme via His 25 (turquoise) and catalyzes its degradation. **b)** Erythrophagocytosis (1) leads to the formation of an erythrophagosome which mediates RBC lysis (2). Via HRG-1 (orange) heme is delivered into the cytosol (3). The liberated heme inhibits the transcription factor Bach-1 (dark green; (5)), leading to the expression of HO-1 (grey). **b, c)** Subsequently, heme is degraded, releasing Fe^{2+}, CO, and biliverdin. Biliverdin is converted to unconjugated bilirubin. In the blood stream, it is scavenged by albumin and transported to the liver, where it is conjugated and further degraded, finally leading to excretion. In contrast, Fe^{2+} is recycled for heme synthesis and, subsequently, erythropoiesis. Different proteins are involved here: Ferroportin (FP) manages the export of Fe^{2+} into the circulation, where it is bound by plasma transferrin (yellow) and distributed to respective sites, such as the bone marrow. In case of superfluous Fe^{2+}, it is stored by ferritin in the cytosol (not shown). However, iron overload leads to an accumulation within the plasma with detrimental consequences.

peptide 2 (OATP2).[82] Within hepatocytes, bilirubin becomes conjugated with glucuronic acid to bilirubin glucuronides, catalyzed by the enzyme uridine 5′-diphosphoglucuronosyltransferase 1.[83] Conjugated bilirubin is more polar than unconjugated. Thus, secretion into the bile and intestine is facilitated. Bacterially controlled, bilirubin is degraded to urobilinoids and finally excreted in feces as stercobilin or urobilin in urine.[84]

The liberated iron (II) ions (Fe^{2+}), however, are not excreted but recycled. Upon release, Fe^{2+} is exported into the blood stream via the transmembrane iron exporter ferroportin (Figure 2b).[85] Subsequently, the plasma ferroxidase ceruloplasmin oxidizes Fe(II) to Fe(III), thereby allowing for the binding to apotransferrin.[86] As a consequence, transferrin delivers Fe(III) to different tissues, such as the bone marrow or liver, allowing for heme biosynthesis and subsequent incorporation in proteins.

Contrary to previous assumptions, very recently a role for *hemolysis* (cf. Chapter 2.2.) in the turnover of senescent RBCs was demonstrated.[87] On the surface of intact senescent RBCs, activation of the Lutheran/basal cell-adhesion molecule (Lu/BCAM) has been recognized, which allows for the adhesion to laminin-α5 of the endothelium in the splenic red pulp under shear stress. This process was accompanied by cell shrinking and the formation of RBCs lacking hemoglobin (*RBC ghosts*).[87] Ultimately, RBC ghosts were phagocytosed by the red pulp macrophages, suggesting hemolytic processing of senescent RBCs prior to intracellular digestion in macrophages.[87]

2.2 Hemolysis

The term *hemolysis* commonly refers to the pathophysiological process of RBC destruction, characterized by a shortened life span (e.g., ~29 days in case of hemolytic anemia[61]). Hemolysis may either occur in the blood vessels or in the RES of different organs (e.g., liver, spleen, bone marrow), and, thus, is subdivided into *intravascular hemolysis* and *extravascular hemolysis*, respectively.[6,88]

Extravascular hemolysis results either from intrinsic or extrinsic defects of RBCs. The most frequent intrinsic defects are due to inherent or acquired anomalies of the RBC membrane (e.g., spherocytosis or in paroxysmal nocturnal hemoglobinuria (PNH)), the hemoglobin structure (e.g., in β-thalassemia and SCD) or the internal metabolism (e.g., pyruvate kinase or glucose-6-phosphate dehydrogenase (G6PD) deficiency).[6,89,90] In contrast, causes for extrinsic defects include infections (e.g., malaria, bartonellosis), hyperactivity of the RES (hypersplenism), mechanical trauma, immunological anomalies

(e.g., autoimmune hemolytic anemia (AIHA)) and toxins (e.g., copper, snake venoms)).[6] Most of these defects follow the predominant mechanism of the induction of RBC deformation or the opsonization with antibodies or complement factors. As already described (cf. Chapter 2.1; Figure 2b), this finally results in the inability to pass the splenic cords and RBCs are phagocytosed. Hence, extensive clearance of defective (in addition to senescent) RBCs is required. Not only the spleen but in particular the liver and the bone marrow support RBC removal under conditions of extravascular hemolysis. Just recently, it was shown that under hemolytic conditions with elevated erythrophagocytosis, liver macrophages turn into an antiinflammatory phenotype, which is directly induced by heme, thereby preventing from toxic effects of the RBC content.[91] At a certain state, transferrin as the iron-transport protein in the plasma (cf. Chapter 2.1) becomes saturated (*iron overload*), leading to the formation of non-transferrin iron(III) ion complexes and a pool of labile iron, as shown after transfusion of stored RBCs[92] or in β-thalassemia, for example.[93] Thus, symptoms of patients undergoing extravascular hemolysis are mainly related to the iron overload. Major clinical biomarkers for extravascular hemolysis are the percentual saturation of transferrin and the level of serum iron as well as the level of unconjugated bilirubin (Figure 2b, c; cf. Chapter 2.1).[94,95]

In comparison to extravascular hemolysis, intravascular hemolysis is more harmful due to the excessive, rapid release of RBC content into the circulation with the possibility to interact with all of the vascular components. For example, in the context of AIHA, it has been proposed that intravascular hemolysis causes a ~10-fold greater RBC destruction than extravascular hemolysis.[96,97] However, depletion of the RES capacity in extravascular hemolysis can lead to simultaneous occurrence of intravascular hemolysis as well (e.g., in AIHA and SCD). Independently, intravascular hemolysis may arise in alloimmune hemolytic anemias, such as upon transfusion reactions, or as a sequel of osmotic and mechanical injuries (e.g., in thrombotic microangiopathy (TMA) or disseminated intravascular coagulation (DIC)).[6,98] Thereby, defects of RBCs are provoked by either surface coating of the RBC membrane, osmotic lysis or mechanical destruction. In the course of RBC rupture, the entire content, including up to 250 trillion hemoglobin molecules for each RBC, are released into the bloodstream (Figure 1; cf. Chapter 2).[5] There, cell-free hemoglobin dissociates into αβ dimers, resulting in an equilibrium between the dimer and tetramer.[99] Subsequently, the dimer is trapped by the acute phase protein haptoglobin (~100 kDa; plasma concentration: ~5-30 μM; derived from reference 100) with very high affinity (estimated K_D ~1 fM[101]), shifting the equilibrium towards the dimeric form of hemoglobin (Figure 3).[100,102,103] Haptoglobin occurs in different phenotype variants: Hp1-1, Hp2-2, and Hp1-2.[100,103,104] All of them consist of two light (α-) and two heavy (β-) chains that are connected by disulfide bridges and differ in the composition of their light chains as well as the tendency to polymerize (Hp2-2 and Hp1-2). As recently shown, each form of

haptoglobin dimer tightly binds one hemoglobin dimer, also in the respective oligomeric states but with different affinities (no precise data available).[105] Correspondingly, a hemoglobin-binding capacity of ~3-18 μM was reported.[100] Upon binding, haptoglobin prevents from renal filtration of hemoglobin and protects the vasculature from cell-free hemoglobin-driven toxicity.[106,107] Consequently, haptoglobin knock-out mice suffer from immense tissue damage upon induction of severe hemolysis.[108] Recently, haptoglobin therapy was shown to protect from hemoglobin-triggered neurological damage after subarachnoid hemorrhage[109,110] and from side effects upon transfusion of stored RBCs.[111] Consequently, it is not surprising that there are currently preclinical trials (phase 1) with haptoglobin 1-1 (CSL888) for the treatment of aneurysmal subarachnoid hemorrhage (CSL Behring), whereas plasma-derived haptoglobin is approved for the treatment of severe hemolysis in Japan since 1985 (Benesis Corporation).[112]

The hemoglobin-haptoglobin complex is then captured by CD163 (K_D ~1-19 nM), a receptor on the surface of circulating monocytes and macrophages.[113–115] Non-complexed hemoglobin showed weaker affinity for binding to CD163 (K_D ~159 nM).[115] After binding of the complex, CD163 initializes internalization of the complex into the macrophage cytosol, where the breakdown of hemoglobin is mediated by proteases and the heme oxygenase system (cf. Chapter 2.1, Figure 2).[113] Interestingly, just recently, haptoglobin degradation fragments were suggested to be used for early biomarkers in TMA related to hematopoietic stem cell transplantation.[116] Moreover, there is also evidence for the expression of CD163 in neurons after intracerebral hemorrhage (ICH)[117], which might support the preventive role of haptoglobin against hemoglobin-triggered neuronal toxicity.[109,110]

Under conditions of severe hemolysis, this scavenging system becomes overwhelmed, resulting in the accumulation of large amounts of cell-free hemoglobin within the plasma. Hemoglobin is then oxidized to methemoglobin by nitrogen monoxide (NO) or other physiologic oxidants.[118,119] Thereby, the extensive release of the heme group is promoted, which results in a pool of extracellular *labile heme* within the vascular compartment. *Labile heme* is defined as being "*buffered by exchange-labile small molecules, peptides, or proteins*" (Reddi, A. R. & Hamza, I. (2016)[120]). Binding to other molecules occurs in a transient fashion.[120,121] In this way, labile heme can affect various molecules and reactions, which leads to the propagation of several pathophysiological processes under hemolytic conditions (cf. Chapter 2.3). However, there is a preventive heme-scavenging system that immediately come into play when heme is released into the plasma. It detoxifies heme by complexation and consists of several heme-binding proteins that encompass α1-microglobulin, α1-proteinase inhibitor (= *α1-antitrypsin*), hemopexin, HSA, and different lipoproteins (Table 1, Figure 3).

Table 1: Heme-binding capacity of known heme scavengers.

Protein*	Suggested binding site (protein:heme ratio)	Heme-binding affinity	Year/Reference
LDL (~3 mM)	- (1:1)	~49 nM	1998[123]
	- (1:2)	~0.83 nM, ~7.7 nM	1999[124]
HDL (~1.4 mM)	- (1:1)	~291 nM	1998[123]
	- (1:2)	~0.53 nM, ~3.4 nM	1999[124]
HSA (~600 μM)	- (1:(>)2)	-	1950[125]
	1:>1	~20 nM (high-affine site)	1974[126]
	fragment aa 124-298 (-)	-	1977[127]
	His residue (1:1)	~9 nM	1980[128]
	- (1:1)	~1 μM	1998[123]
	- (1:1)	~0.5 nM	1999[124]
	His 146 [FA1B domain] (1:1)	-	2003[129]
	His 146 [transient], Tyr 161 [irreversible] (1:1)	~40 μM	2005[130]
	FA1 domain (1:1)	~500 nM	2007[131]
	His 146 (1:1)	-	2012[132]
	- (1:1-2)	~12.6 nM	2012[122]
α1-PI (≤44 μM)	close to Trp194 (1:1)	~20 nM	2012[122]
hemopexin (~17 μM)	- (1:1)	-	1964[133]
	- (-)	≤10 nM	1972[134]
	- (1:1)	~5.3 fM	1974[135]
	His 79**, His 150** (1:1)	-	1991[136], 1993[137]
	His 79**, His 150**	30 nM (rHx)	1994[138]
	bis-histidine with His 150** (1:1)	-	1995[139]
	His 236** and His 293**; His 105** and His 150** (1:(>)1)	-	1999[140]
	- (1:1)	~15 pM	1999[124]
	- (1:2)	-	2000[141]
	potential transient binding, recruiting sites: His 260, His 238 (1:>1)	~320 pM (high affine site), ~3.5 μM (all binding sites)	2020[142]
	His 150** as 2nd binding site suggested (1:>1)	-	2020[143]
α1-MG (~2.5 μM)	- (-)	2.4 μM (free), 1.6 μM (+IgA)	2004[144]
	Cys 34 [CP] (1:1)	-	2012[145]
	- (1:2)	-	2012[146]
	Cys 34 [CP], His 123 (1:2)	~7 μM, 190 μM	2014[147]
	Cys 34 [CP], His 123 (1:2)	~14 μM	2016[148]

*Proteins are depicted in plasma concentration-dependent hierarchy. **Numbers are adjusted to positions according to mature human hemopexin. aa: amino acid; FA1: fatty acid binding site 1; FA1B: fatty acid binding site 1B; HDL: high-density lipoprotein; HSA: human serum albumin; IgA: immunoglobulin A; LDL: low-density lipoprotein; α1-MG: α1-microglobulin; α1-PI: α1-protease inhibitor; rHx = recombinant hemopexin.

Moreover, immunoglobulins were suggested as heme scavengers.[10] Studies on a potential transient binding of heme by the hemoglobin scavenger haptoglobin revealed either very weak or no binding at all.[122]

Different lipoproteins, more precisely low-density lipoprotein (LDL; ≤4800 kDa[149]; serum concentration (LDL-cholesterol): ~3 mM[150]), very low-density lipoprotein (VLDL; ≤1000 kDa[149], serum concentration (VLDL-cholesterol): 0.5 mM[150]), and high-density lipoprotein (HDL; ≤360 kDa[149], serum concentration (HDL-cholesterol: ~1.4 mM[150]) that usually deliver lipids in the circulation, were reported to bind heme with rather high affinity. Thereby, one (K_D of ~49 nM (LDL); K_D ~291 nM (HDL)) to two (K_{D1} of ~0.83 nM and K_{D2} of ~7.7 nM (LDL); K_{D1} ~0.53 nM and K_{D2} ~3.4 nM (HDL)) heme molecules bind to the superficial lipid monolayer of the proteins.[123,124] However, in the presence of ROS, heme induces lipoprotein oxidation, which might contribute to artherogenesis.[123] α1-microglobulin as well as hemopexin were reported to protect lipoproteins from heme-triggered oxidation suggesting a superior role for these heme scavengers.[151,152] As one of the most abundant (plasma concentration: ~600 μM[153]) proteins in human blood, HSA (67 kDa) was already found in complex with heme under excessive hemolytic conditions in 1941.[154] Since then, it was analyzed for its heme-binding properties several times (Table 1). The affinity for heme was determined to be within the nano-/micromolar range (ranging from 0.5 nM to 40 μM; Table 1).[122–124] These differences might be due to different affine heme-binding sites in albumin, whose existence was already suggested in 1973.[126] While the high affinity binding site is specific for heme, other sites might be also used for the transport of fatty acids.[155] Transient heme binding happens very fast to the surface of HSA via His 146, which is followed by an internalization of heme towards a more buried heme-binding site, Tyr 161, with slower kinetics.[128,130] Usually responsible for the inactivation and, thus, prevention from inflammatory enzymes, the acute phase protein α1-proteinase inhibitor (~52 kDa, plasma concentration: ≤44 μM) has been shown to bind heme with a very similar affinity (K_D ~20 nM; stoichiometry: 1:1) as HSA.[122] The heme-binding site was suggested to be close to Trp194 (as only derived from albumin). Moreover, a heme-recruiting mechanism from unspecific binding sites towards the high-affinity binding site was proposed.[122] *In vitro* experiments demonstrated the capability of α1-proteinase inhibitor to prevent heme-triggered neutrophil adhesion, ROS production, and proinflammatory responses.[156] Hence, its therapeutic potential, such as after lung transplantation, was emphasized.[157] Furthermore, it is currently in the phase of clinical development for the administration after allogenic hematopoietic stem cell transplantation (CSL964®, CSL Behring) and already on the market for the compensation of inherited deficiency (e.g., Respreeza®, CSL Behring).

The lipocalin α1-microglobulin (~26 kDa; serum concentration: ~2.5 μM (total α1-microglobulin; derived from reference 158)) has been proposed to possess two heme-binding sites, a higher affinity (K_{D1} ~7 μM; Cys 34) and a very low affinity (K_{D2} ~190 μM; His 123) binding site.[145–148] As α1-microglobulin is partially (50%) circulating in complex with IgA, unaltered heme binding of this construct was also demonstrated.[144] Its relevance as a potent heme and radical scavenger is evidence by α1-microglobulin knock-out mice.

These exhibit massive hemolysis.[159] Interestingly, elevated levels of α1-microglobulin in case of the saturation of haptoglobin and hemopexin were recognized, suggesting a compensation mechanism of the scavenging systems.[160,161] Under these conditions, α1-microglobulin transports heme into the kidneys, thereby causing acute kidney injury.[161] Recently, several patents were submitted that suggest α1-microglobulin as a therapeutic agent for the protection of hematopoietic stem cells in the bone marrow (e.g., in chemotherapy) or as a common radical scavenger in several diseases, such as arthritis or ischemia/reperfusion-related diseases.[162,163]

Hemopexin (~57 kDa, plasma concentration: ~17 μM[164]) is handled as the main scavenger for heme under hemolytic conditions due to its outstanding high heme-binding affinity in the femto-/picomolar range (K_D of ~5.3 fM[135]; ~15 pM[124]; 1:1). Several potential candidates for the actual heme-binding site were suggested (Table 1). Just recently, heme-binding to hemopexin was reevaluated, confirming a high heme-binding affinity (K_D of ~320 pM[142]) but a stoichiometry of one molecule hemopexin binding more than one heme molecule.[142,143] A recruiting mechanism from the residues His 260 and/or His 238 by transient binding of heme was suggested that subsequently leads to the internalization of heme towards the bis-histidine hexacoordination with His 236 and His 293.[142] Recently, it was demonstrated that hemopexin participates in heme scavenging with an equimolar heme-binding capacity, while HSA could bind the double amount of heme.[164,165] In fact, hemopexin was shown to effectively prevent from heme-driven complement activation in hemolysis,[166] from cancer progression[167] and from acute kidney injury in SCD[168]. Thus, hemopexin (CSL889®; CSL Behring) is currently in phase 2 trials for the treatment of SCD. Finally, the quantity of the scavenger proteins in plasma as well as their heme-binding affinities suggest a potential temporal chronology. It is believed that most of the heme is bound by lipoproteins in the first instance due to their plasma levels, high heme-binding affinities and faster association constants.[124] However, a transfer to other plasma proteins is necessary to prevent from heme-driven oxidative modification of lipoproteins. It is therefore taken over by HSA, which, in turn, transfers heme to hemopexin.[142,169] Subsequently, hemopexin delivers heme to the LDL receptor-related protein 1 (LRP1)/CD91 (K_D ~0.3-0.5 nM[170]), which is found on the surface of several cell types, among them macrophages and hepatocytes.[170] After endocytosis, heme is degraded by the heme oxygenase system. In contrast, hemopexin has been shown to be either recycled and intactly released back into the plasma[171] or degraded in lysosomes.[170] Up to date, only this way of cellular heme uptake in intravascular hemolysis has been reported. Cellular uptake of the heme-albumin complex upon binding to the transferrin receptor 1 (TFR, CD71), followed by the HO-1-mediated degradation of heme, was just recently discovered.[172] The affinity of the heme-albumin complex for binding to CD71 was characterized by a K_D of ~0.75 μM.[172] However, a role for this process in the context of hemolysis has not been described yet.

The other proteins might play a subordinate role and bind heme in case of massive heme overload. In fact, it was demonstrated that ~44% of labile heme becomes bound to HSA, ~35% to hemopexin, 18.6% to HDL and 2.4% to LDL in an equilibrium state.[124] Other proteins were not considered at that time. The heme-binding capacity of normal serum was demonstrated to allow for buffering of 1.8 mM heme.[164] Considering the amount of hemoglobin, consequently each ruptured RBC releases approximately one billion heme molecules, which roughly corresponds to a concentration of 80 mM (Figure 1).[4,5] Therefore, it is not surprising that in most severe cases of intravascular hemolysis even the described protective systems become overwhelmed. In this context, labile heme levels of 2->350 μM were reported[173–180] that might remain for an interaction with other molecules (such as proteins) and, thus, exert toxic effects (cf. Chapter 2.3). However, there is a lack of adequate standard methods for the quantification of labile heme under these conditions.[181]

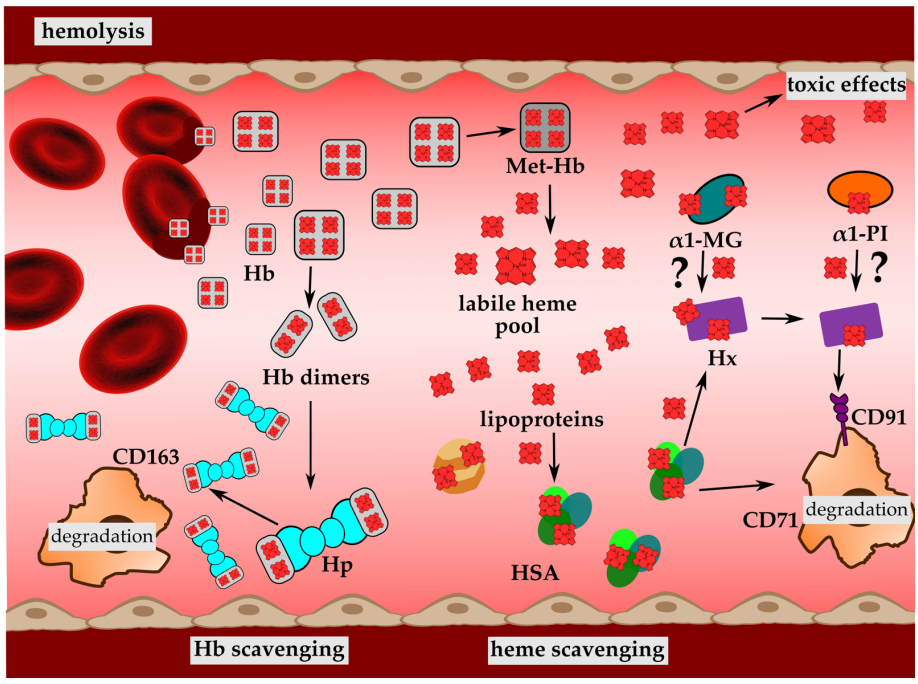

Figure 3: Overwhelming of the natural heme scavenging system in intravascular hemolysis. Upon RBC rupture, hemoglobin (Hb) is released within the intravascular space. Haptoglobin (Hp) captures Hb dimers and subsequently binds to the scavenger receptor CD163, which mediates the internalization of the haptoglobin-hemoglobin into macrophages. As a consequence, hemoglobin is degraded. When haptoglobin becomes exhausted, cell-free hemoglobin accumulates and is readily oxidized to methemoglobin (Met-Hb), which releases its heme group, leading to a pool of labile heme. Labile heme, in turn, is trapped by different scavenger proteins, including lipoproteins (e.g., LDL), human serum albumin (HSA), hemopexin (Hx), α1-microglobulin and α1-proteinase inhibitor. A final transfer to the high-affinity heme-binding protein hemopexin is assumed. The hemopexin-heme complex is internalized into macrophages via CD91. Moreover, the albumin-heme complex can be trapped by the transferrin receptor CD71. Both leads to the degradation of heme via the heme oxygenase system. In case of excessive intravascular hemolysis, even these scavenger systems become overwhelmed leading to the accumulation of high amounts of labile heme, which exerts toxic effects.

2.3 Pathophysiological potential of labile heme

In hemolytic disorders, heme has been linked to prooxidant, cytotoxic and proinflammatory responses and is thus considered as an damage-associated molecular pattern (DAMP).[6–11]

Heme induces oxidative stress by different mechanisms that overall culminate in ROS generation. Due to its central iron ion, heme is able to catalyze Fenton-type reactions leading to the generation of free hydroxyl radicals as potent oxidants.[182,183] It can further act as a pseudo-peroxidase and promote hydroperoxide conversion, which produces alkoxyl and peroxyl radicals.[184] Moreover, heme evokes intracellular, more precisely mitochondrial, ROS generation, via enzymes like NADPH oxidase or spleen tyrosine kinase (Syk).[9,185,186] Heme-triggered ROS formation has detrimental consequences. It can lead to damage of cellular components, such as lipids,[187] DNA,[188] and proteins[189]. Lipid peroxidation may even amplify ROS generation. Moreover, heme is capable of intercalating within membranes.[7,190] Together with the oxidation of phospholipids, it results in membrane injury through phospholipid damage and permeability impairment.[191] As a consequence, proinflammatory and apoptotic cell signaling is initiated. In this way, heme is capable of propagating and potentiating its own release into the vascular compartment by further triggering hemolysis. The intercalation and accumulation of heme into the RBC membrane as well as the direct interaction of heme with the cytoskeletal protein spectrin leads to conformational changes and subsequent RBC rupture.[192–195] In addition, heme has been shown to cause ferroptosis of platelets as well as necroptosis and apoptosis of endothelial cells.[19,196,197] However, heme-induced oxidative stress is in particular recognized in the kidneys, as heme triggers oxidative stress in the epithelium leading to inflammation and renal failure.[198] Moreover, it is, for example, involved in the pathology of arteriosclerosis, SCD and Alzheimer's disease.[199–201]

In hemolytic disorders, it is assumed that heme attacks the endothelium right after its release.[6,11] Initial heme-induced activation of endothelial cells serves as a basis for the initiation and potentiation of the triad of inflammation, complement system and blood coagulation system. Thereby, RBC-derived MVs might assist in delivering heme to the endothelium.[202] Interaction of heme with the endothelium rapidly impairs endothelial barrier integrity, permeability and consequently provokes endothelial barrier dysfunction.[203,204] These effects were recently attributed to the activation of the p38 mitogen-activated protein kinase (MAPK)/heat shock protein 27 (HSP27) pathway by heme and correlated with hemolysis-associated pulmonary hypertension.[204,205] Moreover, reduction of important tight junction proteins (e.g., claudin 1 and 5) was observed.[204] Heme-triggered

endothelial activation is also accompanied by enhanced expression of adhesion proteins, such as intracellular adhesion molecule 1 (ICAM-1), P-selectin, and vascular cell adhesion molecule 1 (VCAM-1),[206–208] which is caused by activation of the Toll-like receptor 4 (TLR4) and subsequent transcription factor (e.g., NF-κB) activation.[209,210] These allow for the rapid adhesion of other cells, like neutrophils, to the endothelium. Before neutrophils can attach, they need to express complementary adhesion proteins on their surface. This was also shown to be mediated by heme in a ROS- and protein kinase C (PKC)-dependent manner.[211,212] The neutrophil cytoskeleton was affected by heme allowing for neutrophil migration into parenchymal tissue.[211,212] Moreover, neutrophils generate neutrophil extracellular traps (NETs) upon exposure with heme, which happens ROS-dependently but TLR4-independently and contribute to the proinflammatory response.[213] Through inhibition of neutrophil apoptosis by addressing the Ras-Raf-MAPK and phosphoinositide 3-kinase (PI3K) pathways, heme ensures the persistence of these cells and maintains their proinflammatory functions.[214] In addition, specific cell types were reported to express more CD11b (granulocytes, monocytes),[215] IL-1β (macrophages),[216–219] IL-8 (granulocytes, endothelial cells, neutrophils)[211,220], keratinocytes-derived chemokine (KC; macrophages)[221], leukotriene B4 (LTB4; macrophages),[222] and tumor necrosis factor α (TNFα; macrophages)[221] upon activation by heme. Heme effectively upregulates also other proinflammatory markers, such as IL-6, chemokine (C-C motif) ligand 2 (CCL2), CCL3, interferon γ (IFNγ), metalloproteinase 8 (MMP8), MMP9, interleukin 1 receptor antagonist (IL-1Ra).[215,223] Recently, even direct heme binding to transmembrane TNFα has been demonstrated. However, the physiological consequence thereof is not yet investigated.[224]

So far, it has been demonstrated that these heme-driven proinflammatory responses are mainly induced by the activation of TLR4 or the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome. Thereby, activation of the TLR4-dependent pathway seems to occur CD14- and myeloid differentiation factor 2 (MD2)-dependently.[215,221,225] Moreover, heme-TLR4 signaling leads to MyD88 and NF-κB activation, and finally to cytokine secretion (e.g., TNFα and KC).[210,221] In this way, heme has been demonstrated to induce inflammatory injury upon ICH (Chapter 2.2) in a TLR4-dependent manner by the release of cytokines, such as IL-1β.[226,227] Blocking of TLR4 prevented from heme-driven inflammation.[227] A TLR4-inhibitor (i.e., TAK-242) was thus suggested for therapeutic application in SCD.[210] However, direct binding of heme to TLR4 has not been validated yet and the assumptions of an interaction is purely based on results gained under conditions of TLR4 deficiency or blocking.[228] In contrast, heme binding to MD2 (Table 2), which is associated with TLR4 and supports the response to its agonist lipopolysaccharide (LPS), was recently demonstrated.[225] Beside a direct induction of TLR4 signaling, also ROS-driven TLR4 activation might be conceivable, as suggested in hemorrhagic shock.[229] Thereby, Syk phosphorylation regulates ROS formation via PKC and phosphoinositide

phospholipase Cγ (PLCγ) signaling.[186] However, the interrelations and interactions within the TLR4 signaling pathway that might be triggered by heme are not entirely understood and need further investigation. Furthermore, there might be a role for heme as an agonist of other TLRs as well. For instance, heme was described to induce MMP9 activity and secretion of different cytokines (i.e., chemokine (C-X-C motif) ligand (CXCL) 1, CXCL2, IL-1β, IL-6, TNFα) via TLR2 in astrocytes, which resulted in brain injury after ICH.[230] Moreover, synergizing with agonists of different other TLRs has been suggested to play a role in the heme-induced cytokine production in macrophages.[186]

For heme-mediated activation of the NLRP3 inflammasome prior ROS generation and priming with LPS is necessary.[217,231] Further, Syk phosphorylation and the NADPH oxidase 2 might be involved.[217] In contrast to TLR4 signaling, IL-1β production and processing is possible by heme-induced NLRP3-signaling.[218,219,221] Thereby, heme was suggested to activate the P2X receptor and subsequently the NLRP3 inflammasome to produce caspase 1, which promotes the release of IL-1β.[218,219] At present, there are several controversial discussions about the exact mechanism on the molecular basis.[9,216,218,219]

The role of heme as an actor in inflammation is probably the best known and explored.[9,232] However, heme possesses antiinflammatory functions as well, thereby suppressing immune activity.[233,234] As such, heme was shown to induce the PI3K/protein kinase B (Akt) pathway in astrocytic glia cells and bind to Aβ oligomers. This, in turn, results in the protection of the glia cells from proinflammatory injury through the reduction of the cytokine expression levels (e.g., CCL5, GM-CSF, and IL-1β) and an impaired uptake of Aβ, which might play a role in the pathogenesis of Alzheimer's disease.[233] Moreover, direct heme binding to interleukins 36α, β, and γ (IL-36α, IL-36β, IL-36γ), yet the only known heme-binding interleukins (Table 2), led to the inhibition of the interaction with their receptors in synovial fibroblasts and, thus, finally abolished proinflammatory response leading to a reduced expression of IL-6 and IL-8.[234] The pathophysiological role of these interactions is not known so far.[234]

In addition, heme was introduced recently as an inducer of the innate immune memory.[235] The observed training of the immune system was caused by the activation of the Syk/c-Jun N-terminal kinase (JNK)-pathway, and was effective in protecting mice from bacterial sepsis.[235]

Simultaneously with the initiation of proinflammatory responses, the complement system is activated in a rapid and efficient manner, either through the classical, lectin or alternative pathway.[236] Heme is capable of activating the alternative complement pathway by promoting complement component 3 (C3) activation, eventually resulting in the deposition of the activation fragments C3a, C3b, C3dg, and iC3b on RBCs as well as C3b and Cb9 on endothelial cells.[208,237–240] Moreover, heme exposure leads to increased serum concentration of C3a, the membrane attack complex (MAC) and C5a.[208] Direct heme binding

to C3, C3a, factor B, and factor H was demonstrated (no K_D values given), which possibly drives the observed effects.[208] For C3 three potential heme-binding sites were suggested (Table 2). Furthermore, formation of the C3 and C5 convertases with heme-exposed C3 was enhanced. Endothelial cells, in turn, expressed less amounts of important complement regulating proteins (i.e., the decay-accelerating factor (DAF) and membrane cofactor protein (MCP)) and more of the adhesion protein P-selectin as a complement activating factor in a TLR4-dependent fashion.[208,239]

In contrast, heme binding to the complement component 1q (C1q; K_D ~0.021-2 μM[40,241]) results in the inhibition of the classical complement pathway by abolishing the interaction of C1q with, for example, C-reactive protein (CRP).[241,242] Two binding sites, Tyr 122 in chain A and Trp190 in chain C, in globular C1q were suggested.[241] The alternative pathway is the most dominant pathway of the complement system. Thus, the effect of heme on C1q is of subordinate nature but might support focusing on heme's impact on the alternative complement system and prevent from unspecific side-effects.

Overactivation of the complement system is a common feature in hemolytic disorders, such as SCD,[243] hemolytic transfusion reactions[244] or atypical hemolytic uremic syndrome (aHUS).[197,208] The kidneys are particularly affected by heme-triggered complement activation.[245,246] The treatment with C3 and C5 inhibitors (e.g., eculizumab) therefore was suggested and is currently in use for the therapy of PNH (cf. Chapter 2.2) and aHUS.[197,247,248] Eculizumab but also hemopexin prevent effectively from heme-triggered complement activation.[166,215] Furthermore, an anti-C5 antibody was successfully demonstrated to be suitable for the treatment of hemolytic transfusion reactions in SCD.[249]

The complement system is inevitably linked to the coagulation system (Figure 4).[250] Thrombosis might thus be a consequence of the heme-driven overactivation of the complement system. Furthermore, heme exerts its cytotoxic effects towards cells that participate in the development of prothrombotic reactions, such as platelets and endothelial cells, and directly interacts with coagulation factors as well (cf. Chapter 2.4).

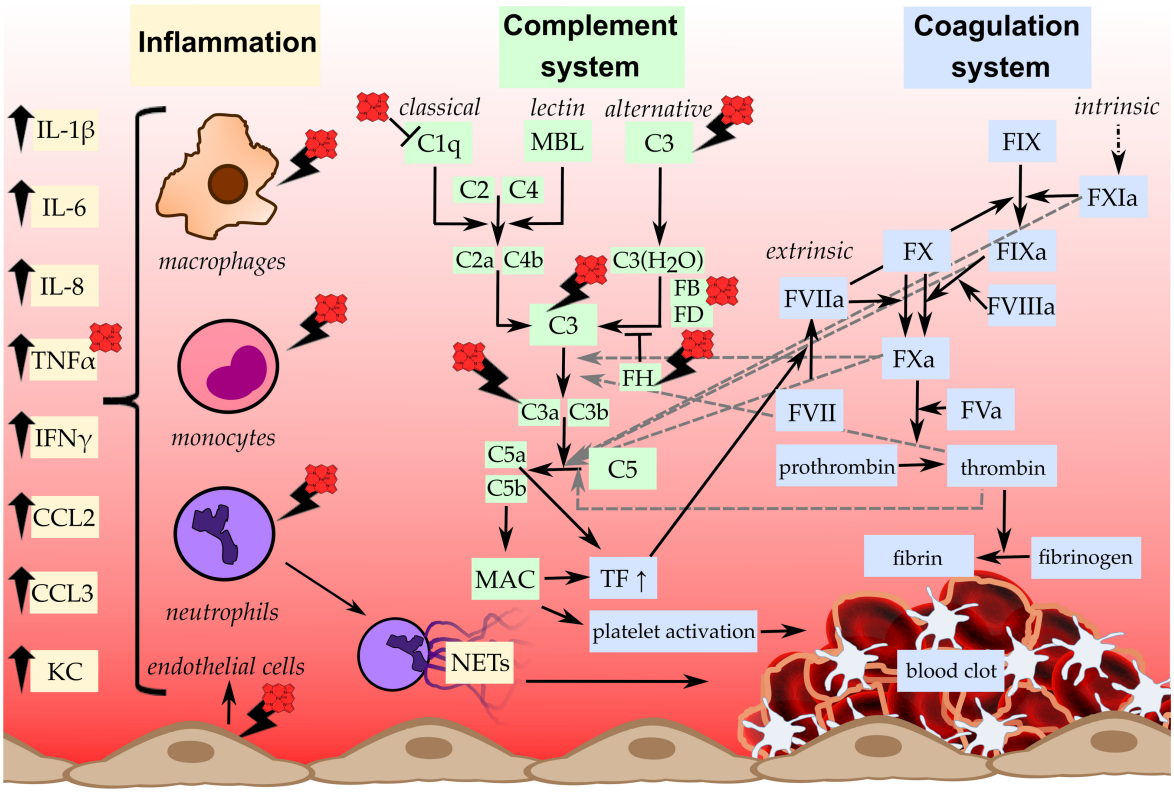

Figure 4: Heme as an actor in proinflammatory and complement system-related reactions and their crosslinking with the coagulation system. Heme is capable of activating proinflammatory pathways (yellow) in different cell types leading to the secretion of various cytokines, such as IL-1β and TNFα (black arrows). Only a selection of cell types, i.e. macrophages (brown), monocytes (pink), neutrophils (violet) and endothelial cells (grey), is depicted. In addition, heme induces NET formation, which recruit proinflammatory cells, RBCs and platelets, and supports clot formation. Direct heme-binding to those upregulated cytokines has not been analyzed yet. In contrast, in particular, the alternative pathway of the complement system (green) is characterized by heme-binding proteins (e.g., C3). Heme activates the alternative pathway by promoting C3(H_2O) generation. As a consequence, the formation of MAC is enhanced, which is able to upregulate TF expression on different cell types as well as to stimulate the activation of platelets.[251,252] Several proteins of the blood coagulation system (blue), such as thrombin and FXa, are known for their complement system promoting activity (grey dashed arrows), thereby again connecting both the coagulation and the complement system.[250,253] In contrast to above mentioned heme-triggered effects, C1q and IL-36 family members (not shown) are inhibited upon heme binding, conferring heme a potential complement-inhibiting and antiinflammatory role. All pathways are not exhaustively illustrated and reduced to main components. C: complement component, C3(H_2O): hydrolyzed C3, CCL: chemokine (C-C motif) ligand, FB: factor B, FD: factor D, FH: factor H, FVa: activated factor V, FVIIa: activated factor VII, FVIIIa: activated factor VIII, FIX: factor IX, FIXa: activated factor IX, FX: factor X, FXa: activated factor X, FXIa: activated factor XI, IFNγ: interferon γ, IL: interleukin, KC: keratinocytes-derived chemokine, MAC: membrane attack complex, MBL: mannose-binding lectin, NETs: neutrophil extracellular traps, TF: tissue factor, TNFα: tumor necrosis factor α.

2.4 Impact of heme on blood coagulation

2.4.1 Thrombosis as an imbalance of blood coagulation

The crucial physiological process that prevents excessive blood loss upon vascular injury, such as a cut, is called *hemostasis*. Under normal conditions it keeps blood fluid, whereas damage of blood vessels induces *blood coagulation*, which ensures rapid closure of wounds through a complex interplay of vascular, cellular, and plasmatic components.[254–259]

Upon endothelial damage, vasoconstriction is immediately initiated, leading to a reduced blood flow velocity at the site of injury and subsequently to an early decrease of blood loss. Injury is accompanied by endothelium activation as well as exposure of subendothelial cells (e.g., smooth muscle cells and fibroblasts) and the extracellular matrix protein collagen. Hence, platelet adhesion starts with the involvement of a variety of receptors and adhesion proteins of the platelet membrane.[254,260,261] The main initial platelet receptor responsible for adhesion to the subendothelial matrix is the glycoprotein (GP) Ib-IX-V complex in combination with the multimeric, mechanosensitive glycoprotein von Willebrand factor (VWF). VWF circulates as a globular multimer (500-10000 kDa) in plasma at a concentration of ~40 nM[262] and can be anchored to the subendothelial collagen. Moreover, it is stretched under shear stress yielding extraordinarily long strings (≤ 1 mm) that allow for the interaction with GPIb-IX-V and primary platelet adhesion.[263,264] Platelets can also bind to collagen via the transmembrane protein GPVI and the integrin $\alpha 2\beta 1$.[265] As a consequence, adherent platelets become activated and secrete thromboxane A_2 as well as the content of α- (e.g., fibrinogen, factor V (FV), factor XIII (FXIII), thrombospondin-1, VWF), and δ-granules (e.g., ADP, calcium(II) ions (Ca^{2+}), serotonin). This further promotes platelet activation, formation of characteristic pseudopods, and the expression of further integrin adhesion receptors (e.g., GPIIb/IIIa, αIIbβ3).[260,266] These allow for the binding of fibrinogen, fibronectin, and VWF, which mediate crosslinking of already adhered platelets and recruit further platelets, leading to platelet aggregation and the formation of the tentative *clot*.[261] Apart from platelets, WBCs (in particular macrophages and neutrophils) contribute to hemostasis.[267,268] They are capable of secreting procoagulant and proinflammatory proteins.

The process of platelet adhesion, activation, and aggregation is referred to as *primary hemostasis*, which forms the basis for the so-called *secondary hemostasis*. Secondary

hemostasis is already initiated in parallel and is amplified by the surface provided through activated platelets.[255] It consists of a network primarily formed by several serine proteases that are consecutively activated. The enzymes circulate in their inactive form in the plasma and are activated by proteolytic cleavage in the cascade, which is either initiated by tissue factor (TF) exposure (former *extrinsic pathway*) or negatively charged surfaces (former *intrinsic pathway*). Activation via the extrinsic pathway might be more important under physiological conditions, while the intrinsic pathway has been suggested to have a supporting role in amplification of the blood coagulation process.[269] Today, the subdivision into intrinsic and extrinsic pathways is mainly used for easier depiction of interrelations (e.g., in case of laboratory testing). The cell-based model rather represents the actual physiological processes and is thus described in the following paragraph.[255]

The model does not contain factor XII, which more recently was discussed to play a more important role in hemostasis than originally proposed with the cell-based model.[270] Thus, in the present thesis, the terms intrinsic and extrinsic pathway are still applied when referring to the activation of coagulation starting from either negatively charged surfaces or from exposed TF, respectively.

Initiation usually occurs on the surface of extravascular cells, including endothelial cells, fibroblasts, macrophages, and monocytes, that constitutively express and expose TF.[269,271] TF binds with high affinity (~45 pM) to activated factor VII (FVIIa) from plasma, forming the *extrinsic tenase*.[272,273] However, there is only a small portion of FVIIa present (plasma concentration: ~72 pM (derived from reference 274), reflecting the need for further activation of FVII (plasma concentration: ~10 nM (derived from reference 262)). It has been shown that TF catalyzes autoactivation of FVII.[275] The usually weak enzymatic activity of FVIIa is strongly increased in complex with TF due to allosteric interactions.[276] Factor IX (FIX; plasma concentration: ~100 nM (derived from reference 262)) and factor X (FX; plasma concentration: ~170 nM (derived from reference 262)) are recognized by the extrinsic tenase and proteolytically activated. In turn, activated factor X (FXa) promotes the activation of its own cofactor factor V (FV; plasma concentration: ~31 nM (derived from reference 262)).[277] Consequently, a small portion of thrombin is generated from prothrombin (plasma concentration: ~1.4 μM[262]) on the subendothelium, which supports platelet activation through binding to protease-activated receptors (PARs) on the platelet surface.[278] Furthermore, thrombin activates FV and FXI, leading to the amplification of the blood coagulation process.[255] On the platelet surface, thrombin is capable of interacting with GPIb-IX-V as well, thereby allowing for the thrombin-driven trapping of circulating FVIII (plasma concentration: ~0.3 nM (derived from reference 262)), which is usually bound to VWF (K_D ~0.21 nM, stoichiometry: 1:50 (FVIII:VWF)[279]) in plasma and released from the complex upon activation by thrombin. FVIIIa then binds with high affinity (K_D ~0.8 nM) to platelets, which possess ~500 binding sites for FVIIIa.[280] By diffusion of FIXa from subendothelial cells

to the platelet surface, it assembles with FVIIIa (K_D ~0.07 nM; *tenase*).[281] Furthermore, FXa binds with high affinity (K_D ~0.03 nM) to its cofactor FVa in the presence of phospholipids (*prothrombinase*).[282] FXI (plasma concentration: ~31 nM (derived from reference 262)) binds to platelets via the apolipoprotein E receptor 2 (ApoER2), which allows for its activation by thrombin.[283] As a consequence, FIX is activated and, again, the tenase and prothrombinase complexes are formed. This enables the propagation of the coagulation process by a massive acceleration of thrombin generation.[255] Intrinsically, these reactions are further triggered by contact activation (for example by collagen exposure), which leads to factor XII (FXII; plasma concentration: ~363 nM (derived from reference 284)) autoactivation. FXIIa, in turn, activates FXI, FVII and prekallikrein (plasma concentration: ~233 nM (derived from reference 285) by proteolytic cleavage, whereas Kallikrein can reciprocally activate FXII. Kallikrein cleaves high molecular weight kininogen (HMWK) as well, which leads to the release of the vasoactive peptide bradykinin, thereby increasing vasoconstriction.[286]

Finally, all of these processes culminate in the generation of the key enzyme thrombin, which is responsible for the conversion of the most abundant clotting factor in plasma (~9 μM[262]), fibrinogen, into fibrin. Fibrin polymerizes to insoluble fibrin and is crosslinked by the transglutaminase activated factor XIIIa (FXIIIa), after activation of factor XIII (FXIII; plasma concentration: ~94 nM (derived from reference 262)) by thrombin.[287–289] Moreover, it was shown that FXIIIa enables the retention of RBCs in thrombi.[290] Hence, secondary hemostasis ensures the stabilization of the primary clot and, thus, wound healing.

Upon completion of wound closure, *fibrinolysis* overwhelms the processes of fibrin clot formation.[291] Plasminogen (plasma concentration: ~2 μM[292]) is activated mainly by the serine protease tissue-type plasminogen activator (t-PA), which is released from endothelial cells (plasma concentration 70 pM[292]). Furthermore, the activation of t-PA and plasminogen to plasmin can be directly mediated by several coagulation factors (e.g., FXIa and FXIIa).[293–295] Plasmin, as a serine protease, cleaves fibrin, leading to the release of fibrin degradation products (e.g., D-dimers).

Usually both, excessive fibrinolysis and blood coagulation, are suppressed by natural antifibrinolytic and anticoagulant proteins, respectively, and are, thus, tightly controlled. For the control of fibrinolysis there are several serpins that irreversibly inhibit t-PA and plasmin (e.g., plasminogen activator inhibitor-1 (PAI-1), α2-antiplasmin or α2-macroglobulin with plasma concentrations of ~0.5 nM (derived from reference 296), ~1 μM (derived from reference 297), and ~4.2 μM (derived from reference 298), respectively. Moreover, the carboxypeptidase thrombin-activatable fibrinolysis inhibitor (TAFI; plasma concentration: ~50 nM[299]) cleaves lysine residues from the carboxy-terminus of fibrin, thereby hindering binding of profibrinolytic proteins to fibrin.[300]

The intact endothelium provides a platform for anticoagulants that allow for the maintenance of blood fluidity. The most important natural anticoagulants are activated

protein C (APC) and antithrombin. The protein antithrombin (AT, plasma concentration: ~3.5 μM (derived from reference 262)) is a serpin and is capable of inhibiting every procoagulant serine protease of the blood coagulation cascade.[301] Its primary substrates are thrombin, factor IXa, and factor X. Its anticoagulant activity is significantly enhanced upon binding of the natural occurring glycosaminoglycan heparin (K_D in nanomolar range[302]),[303] which is why heparin is commonly used to treat or prevent acute thrombotic states, such as venous thrombosis or in ischemic heart diseases.[304,305] AT itself is approved as an anticoagulant drug as well (e.g., Kybernin®, CSL Behring, and AT III NF®, Baxalta).

The second major inhibitor of the coagulation cascade is activated protein C, which involves a set of proteins, the *protein C system* (i.e., thrombin, thrombomodulin (TM), endothelial cell protein C receptor (EPCR), protein S, and protein C). Protein C circulates in its active (APC; 40 pM) and inactive form (70 nM) in the plasma.[306] It is activated in a thrombin-dependent manner.[307] Thrombin binds to the endothelial transmembrane receptor TM (K_D ~6.4 nM[308]) and activates protein C, which is further catalyzed when protein C is bound to its endothelial receptor EPCR (K_D ~29 nM[309]). Subsequently, thrombin becomes rapidly inactivated and released for clearance, thereby regenerating TM for further protein C activation. The generated APC is a serine protease and attenuates blood coagulation through protein S-assisted proteolytic cleavage and, in turn, irreversible inactivation of the important cofactors FVa (K_D ~7 nM[310]) and FVIIIa (K_D ~546 nM[311]) of the tenase and prothrombinase complexes. Moreover, APC was shown to have profibrinolytic, cytoprotective, and antiinflammatory functions.[312,313] APC itself (recombinant form, Drotrecogin alfa) was earlier approved as a drug in 2001 by the FDA for the treatment of severe sepsis (Xigris®, Eli Lilly), but was removed from the market in 2011 due to a lack of efficiency and bleeding as a major risk.[314] At present, there is a single drug available that contains pure plasma-derived protein C (Ceprotin®, Baxter Healthcare Corporation), which is provided for the treatment of the symptoms (such as *purpura fulminans*) of severe congenital protein C deficiency.

Other naturally occurring anticoagulants are for example TF pathway inhibitor (TFPI; plasma concentration: ~2 nM (derived from reference 315) and the heparin cofactor II (plasma concentration: ~1 μM (derived from reference 316).

As many participating components the blood coagulation system involves, so many opportunities for dysregulation are present. Imbalance of blood coagulation, either inherited or acquired, can lead to excessive bleeding (*hemorrhage*; e.g., in hemophilia A or von Willebrand disease (VWD)) or to abnormal, occlusive thrombi formation (*thrombosis*), which can have immediate and serious consequences. Therefore, it is not surprising that thrombosis is one of the leading causes for death worldwide.[317] Thrombosis occurs either in veins or arteries and is thus subdivided in venous and arterial thrombosis.[318]

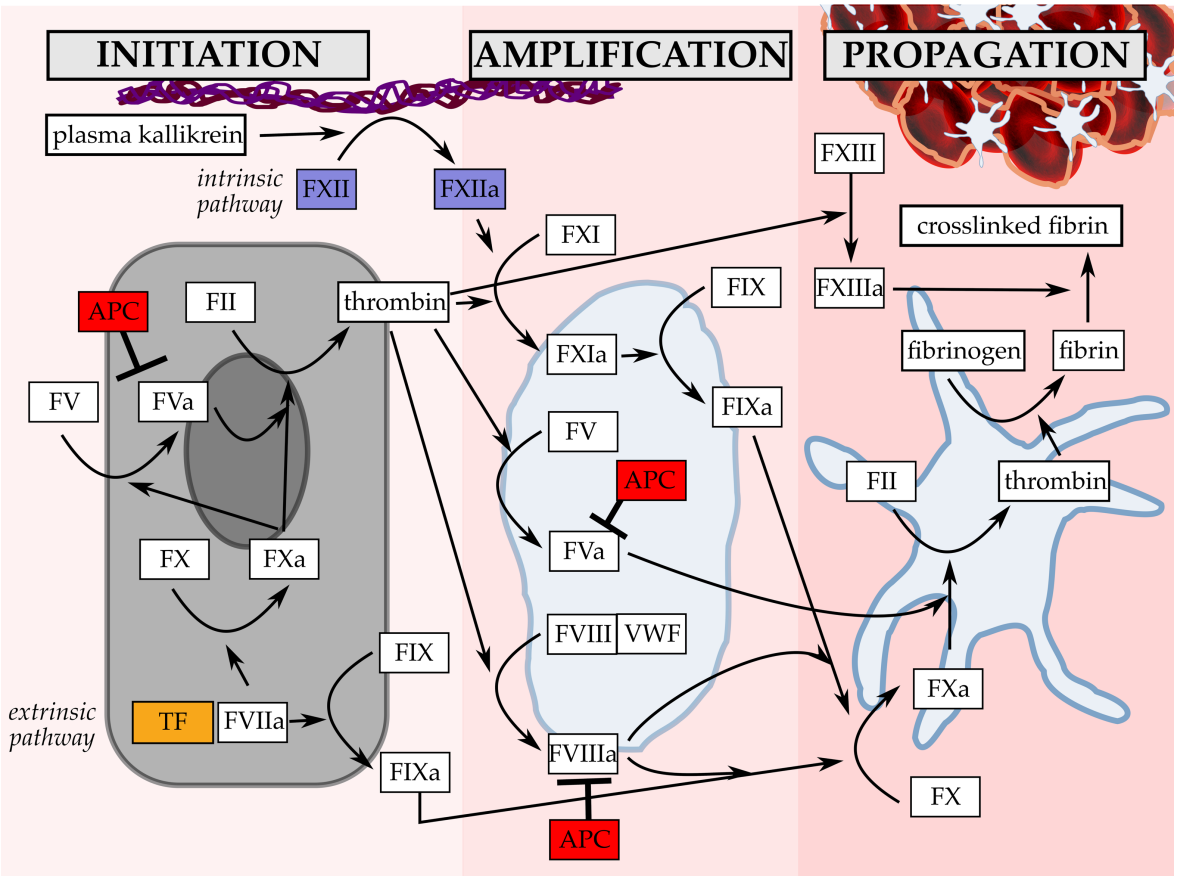

Figure 5: Cell-based model of secondary hemostasis. Secondary hemostasis involves a complex network of enzymes (mostly serine proteases) that trigger initiation, amplification, and propagation of blood coagulation. The initiation is mainly driven by the exposure of tissue factor (TF; orange; *extrinsic pathway*) on different cell types (e.g., endothelial cells). FVIIa can activate FX and FIX thereby mediating amplification. The exposure of negatively charged surfaces, such as subendothelial collagen, may activate the cascade through activation of FXII as well (violet; *intrinsic pathway*). Thrombin, which is also generated in small amounts during the initiation phase triggers the activation of FXI, FV, and FVIII in the amplification phase on the surface of platelets. During the propagation phase the processes finally lead to the generation of crosslinked fibrin, which stabilized the blood clot. Due to the importance for the present thesis, the coagulation inhibitor activated protein C (APC; red) is also shown.

Arterial thrombosis (*atherothrombosis*) usually occurs upon atheroma rupture, whereas venous thrombosis is characterized by fibrin- and RBC-rich thrombi that may be formed also without prior endothelial damage.[318,319] Several risk factors can promote the pathology of thrombosis, including immobility, age, and smoking, but in the end the underlying initial starting point of thrombosis is usually either due to the damage of the endothelium (e.g., after surgery or infection), reduction of blood flow (e.g., after heart failure) or a change of coagulation factors (qualitative or quantitative) (*Virchow's triad*).[320] As a consequence of ischemia, for example, there is a high risk of embolization leading to a thrombus that pass the vascular system. In the worst case, it blocks a vessel and, thus,

reduces or even stops blood flow and oxygen supply to organs (*hypoxia*), such as the heart. Eventually, this provokes myocardial infarction or pulmonary embolism.[321,322] The most common inherited thrombophilic diseases are: 1) factor V Leiden (prevalence in Europe and USA: ~3-8% heterozygote, ~0.02% homozygote), an APC resistance due to a mutation in FV gene, 2) prothrombin mutation (prevalence in Europe and USA: ~1.7-3%) that leads to increased prothrombin levels, 3) moderate protein C deficiency (prevalence worldwide: ~0.2%), 4) moderate protein S deficiency (prevalence worldwide: ~0.2%), and 5) antithrombin deficiency (prevalence worldwide: ~0.02-0.2%).[323]

2.4.2 Hypercoagulability in hemolytic diseases

Apart from these thrombophilias that are directly associated with a higher risk of thrombosis, hemolytic conditions are often accompanied by moderate up to dramatic thrombotic complications, as is exemplified by SCD, aHUS, and PNH (cf. Chapter 1, 2.2 and 2.3) in the following.

One of the most prevalent hemolytic disorders in Europe is SCD (prevalence: 0.01-0.05%).[324] Caused by an autosomal recessive hereditary point mutation of the β-globin gene (β-chain hemoglobinopathy), it leads to the expression of abnormal hemoglobin S (HbS; Gluβ6Val; Figure 6a). Due to the substitution of the polar glutamic acid by a hydrophobic valine residue, the solubility of deoxyhemoglobin is decreased. Thus, hemoglobin polymerization is promoted, which leads to the formation of less flexible fibers.[325] As a consequence, RBCs become more rigid with an elongated shape (*sickling;* Figure 6a) and are prone to burst while passing the circulation (shortened life span of ~15 days), thereby causing chronic hemolytic anemia.[326] Vasoocclusive crisis (VOC) accompanied by inflammation and nociception is the major painful consequence.[327] Development of thrombosis has been observed in the hepatic vein and inferior vena cava in particular, which can extend to the lung vasculature.[328,329] Moreover, there is an incidence of ~11% for VTE in adult SCD patients, which is comparable with inherited thrombophilia, such as protein C deficiency.[16] Sickle cells aggregate, obstruct the vessels and finally abolish oxygen supply, leading to hypoxia, necrosis of tissues as well as to immense pain attacks and, finally, in most severe cases to death.[330] A major cause of death in SCD is stroke.[331]

Typical biomarkers for blood coagulation, such as D-dimers, fibrinogen, VWF, FVIII, thrombin, and thrombin-antithrombin complex are elevated,[332–336] whereas FXII, plasma prekallikrein, HMWK, ADAMTS13, protein S, and protein C activities and/or levels are decreased (Figure 6b).[333,334,337–340] Endothelial dysfunction might be triggered by initial endothelial inflammation, leading to the expression of adhesion proteins.[341]

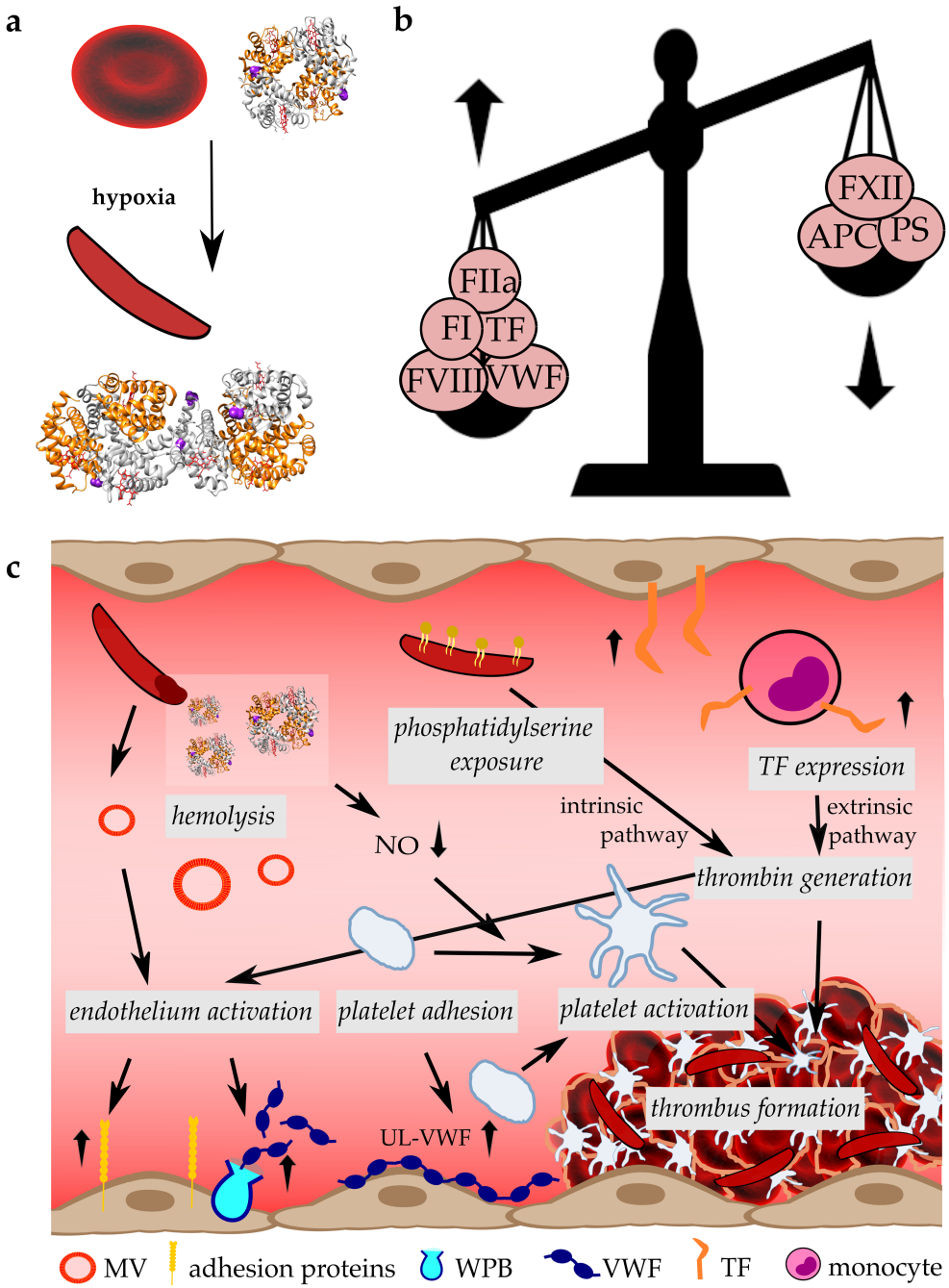

Figure 6: Thrombosis in SCD. a) Upon deoxygenation (in hypoxia), HbS (PDB: 2HBS) polymerizes (dimer depicted) due to a mutation (Gluβ6Val; purple) in the β-chain (orange), forms thick fibers and causes RBC sickling. The shape change finally leads to premature RBC rupture, culminating in hemolysis. **b)** As a downstream event, thrombosis is observed as a common complication in SCD. Plasma levels of several procoagulant proteins such as fibrinogen (FI), thrombin (IIa), factor VIII (FVIII), von Willebrand factor (VWF), and tissue factor (TF) are elevated,[332–336] whereas the plasma concentration of FXII and coagulation inhibitors (e.g., activated protein C (APC) and protein S (PS)) is decreased.[333,334,337–340] **c)** Hemolysis-driven thrombosis has been most studied in SCD. Some basic mechanisms are already known that are directed by endothelial activation, nitrogen monoxide (NO)-scavenging by hemoglobin, phosphatidylserine exposure (e.g., by RBCs and MVs) and elevated TF expression, that finally cause thrombus formation. WPB: Weibel-Palade body; UL-VWF: Ultra-large VWF.

TF is overexpressed on different cell types (i.e., endothelial cells and monocytes) and MVs (mainly from endothelial cells and monocytes), which consequently triggers the extrinsic coagulation activation.[342–347] Some studies suggest a correlation with reperfusion injury, NO scavenging by cell-free hemoglobin and elevated soluble CD40 ligand (sCD40L) from platelets.[332,348,349] Despite its role as a mediator of the plasmatic coagulation, increased thrombin levels in SCD might contribute to the vascular pathology via PAR-1 activation, leading to Weibel-Palade body (WPB) exocytosis and, thus, to release of the adhesion protein P-selectin and the prothrombotic VWF (Figure 6c).[13] Circulating ultra-large VWF (UL-VWF) is significantly increased, which results from a proteolysis resistance towards ADAMTS13 either due to neutrophil-mediated oxidation of Met 1606 (ADAMTS-13 cleavage site) or binding of cell-free hemoglobin.[350–352] UL-VWF promotes platelet and sickle cell adhesion to the endothelium.[353] Apart from the active coagulation factors, also platelets are activated in SCD, which leads to a high aggregation potential.[354] Platelets in SCD are characterized by a higher degree of P-selectin and CD40L expression as well as integrin αIIbβ3-mediated fibrinogen binding.[349,355,356] Furthermore, MVs (mainly RBC-derived) or RBCs that undergo eryptosis provide a suitable surface for FXI- and FXII-dependent thrombin generation (intrinsic pathway) by phosphatidylserine exposure (Figure 6c).[346,347,357] The current trend for the treatment of SCD is focused on hematopoietic stem cell transplantation and gene therapy that both target the disease at its origin.[358,359] Both options are expensive and special devices are required. This hinders the majority of patients to be treated in this way. The administration of drugs that induce fetal hemoglobin (HbF) production, like hydroxyurea, or drugs that prevent from sickle cell dehydration (e.g., clotrimazole), is common.[359] Both strategies attenuate HbS polymerization and premature rupture of RBCs. In order to prevent adhesion of the defect RBCs to the vessel wall, selectin inhibitors (e.g., rivipansel (Pfizer)) were suggested for the treatment of VOC in SCD,[360] but just recently failed in clinical phase 3. Platelet targeting drugs, such as eptifibatide (αIIbβ3-inhibitor), aspirin (cyclooxygenase inhibitor), and prasugrel ($P2Y_{12}$ ADP receptor inhibitor) were not effective in reducing severity of VOC.[361–363] The administration of coagulation factor inhibitors, such as rivaroxaban (FXa inhibitor), warfarin (inhibitor for vitamin K-dependent synthesis of coagulation factors such as FIX), and tinzaparin (thrombin inhibitor) minimized thrombophilia in SCD.[14,364–366] However, the overall drawback of available anticoagulants for the treatment of VOC in SCD is the high bleeding risk.[13]

In contrast to SCD, aHUS and PNH with a prevalence of ~0.005%[367] and ~0.002%[368], respectively, are even less common and, thus, less investigated. aHUS is frequently caused by a gene mutation of a complement factor or regulator and is characterized by a triad of intravascular hemolysis, thrombocytopenia (low platelet count) and acute kidney injury.[369,370] Dysregulation of the alternative complement system leads to the development of TMA (cf. Chapter 2.2) mainly in the kidney. Thereby, TMA has often been associated

with the most procoagulant form of VWF, UL-VWF, whose formation is usually prevented by the metalloproteinase ADAMTS13.[12,371] Due to the complexity and rarity, the pathology of this disease and its molecular basis is less explored and currently under investigation. As such, the participation of hemolysis in TMA as a clinical feature of aHUS is not well understood today, but there is a clear evidence for a role of heme in the development of TMA.[208] Plasma exchange, eculizumab treatment for suppression of the complement reactions or kidney transplantation are applied for therapy of aHUS.[372]

PNH (cf. Chapter 2.2) is an acquired hemolytic disorder that results from a X-linked somatic mutation of the gene for phosphatidylinositol glycan A (PIGA) in the hematopoietic stem cells.[373,374] PIGA is essential for phosphatidylinositol N-acetylglucosaminyltransferase, which is required for the synthesis of the membrane protein anchor glycosyl-phosphatidylinositol (GPI). Consequently, affected cells are deficient of the complement inhibitors DAF and MAC-inhibitor protein (MAC-IP) and, thus, prone to complement activation and complement-induced intravascular hemolysis. In addition, PNH is characterized by thrombophilia, in particular hepatic vein thrombosis (Budd-Chiari syndrome).[375,376] Thereby, thromboembolism appears to be the most common cause for death in PNH (~40-67% of deaths).[15] Promoting factors might include a high amount of antiphospholipid antibodies, the formation of procoagulant platelet vesicles or microparticles, MAC-stimulated prothrombinase activation, an increased interaction of MAC-IP-deficient neutrophils with platelets and endothelial dysfunction.[15,377,378] Furthermore, it has been suggested that the increasing amounts of free hemoglobin (from intravascular hemolysis) might be responsible for the observed thrombotic effects due to hemoglobin-mediated platelet activation, thrombophlebitis, NO depletion (leading to vasoconstriction and platelet activation), inhibition of ADAMTS13, ROS generation, and increase of TF levels.[15] However, there is a need for a detailed analysis of the correlation between intravascular hemolysis and thrombosis in PNH. Since 2007, eculizumab is the only FDA-approved drug for the treatment of PNH and has been shown to successfully prevent from thrombosis in patients, supporting the theory of initial complement activated thrombosis in PNH.[379]

Per definition of the European commission, all of these disorders are considered as rare diseases (prevalence: ≤0.05%; European commission regulation #141/2000). However, taken all patients with inherited and acquired hemolytic disorders together (cf. Chapter 2.2), a larger number of patients are affected. This can be exemplified with the high rate of blood transfusions (~4 million RBC transfusion in 2014[380]) in Germany. It saves life of different recipients, such as patients with SCD (~3 units[381]) or trauma patients (>50 units[382]). Every unit contains approximately ~2 trillion RBCs[383], which roughly corresponds to an amount of 2×10^{21} heme molecules (as derived from reference 3; cf. Chapter 2; Figure 1). Hence, every transfusion involves the risk for transfusion-related hemolysis (e.g., through

incompatibility, device inefficiency or long-term storage of RBCs) and its associated complications, including arterial thrombosis and VTE.[384–389]

2.4.3 Interaction of heme with components of the blood coagulation system

Labile heme has been recognized as a participating component in hemolysis-driven thrombosis as well (as reviewed in the present thesis (cf. Chapter 4.1)). The actual role and, in particular, the complex interrelations of heme-triggered effects are poorly understood. However, it clearly enriches the information on the molecular and cellular basis with respect to the thrombotic state of patients suffering from hemolytic disorders.

Heme-exposed endothelial cells are characterized by cell retraction, increased permeability, detachment, and denudation, which might be due to oxidative stress (lipid and protein oxidation) as well as the activation of apoptotic and/or necroptotic signaling pathways.[196,203,204,390–394] These morphological changes are accompanied by uptake and accumulation of heme in the plasma membrane.[392] Prior to endothelial cell death, mitochondrial intrinsic cell death is provoked, followed by the generation of autophagosomes, which is promoted by increased levels of the microtubule-associated protein light chain (LC3)-II on the endothelial surface as induced by heme.[394] Upon heme-induced activation, endothelial cells express TF in a NF-κB-dependent fashion.[395] Heme binds to and activates also the receptor for advanced glycation end products (RAGE; K_D ~6.8 μM) leading to extracellular signal-regulated protein kinase (ERK) 1/2 and Akt signaling, and finally to TF expression.[396] As a consequence of endothelial activation and denudation, collagen is exposed and platelet adhesion as well as aggregation is initiated.[390,391] These processes are triggered by the elevated expression and exposure of adhesion proteins (i.e., E-selectin, P-selectin, ICAM-1, VCAM-1, and VWF) through activation of TLR4 signaling and degranulation of WPBs by heme.[197,206–210,397] Furthermore, expression of TM in skin and liver is increased as well, but decreased in the lung microvasculature.[197] However, the underlying mechanism is not known so far.

Platelet activation by heme is associated with ferroptotic signaling, culminating in elevated cytosolic ROS levels and subsequent upregulation of glutathione (GSH) as well as lipid peroxidation.[19,398] ROS-dependent inflammasome activation via NLRP3 was reported as the underlying mechanism for heme-mediated platelet activation.[19] Furthermore, platelet activation can occur through direct heme binding to the C-type lectin-like receptor 2 (CLEC2; K_D ~200 nM), which subsequently directs Syk and PLCγ2 signaling.[18] In addition,

researchers proposed that heme triggers TLR4 (MyD88-independent) activation in platelets, leading to mitochondrial ROS production and Ca^{2+} uptake.[399–401] Subsequent secretion of α-granules is accompanied by the release of thrombospondin-1.[400,402] As an antagonist of NO, thrombospondin-1 may promote platelet aggregation as a consequence.[403] Heme also induces the secretion of ADP and serotonin in platelets, suggesting the release from δ-granules as well.[404] Both result in the aggregation of platelets.[401,404] There is also evidence that heme synergizes with the hormone adrenalin to enhance platelet aggregation.[405,406] Thromboxane A_2 release has been associated with heme-triggered platelet aggregation as well.[401]

Despite of effects on the main cellular components, endothelial cells and platelets, heme is capable of interacting with RBCs and neutrophils in a way that might promote coagulation processes. In particular, RBC-derived MVs accumulate a significant portion of labile heme within their membrane (approximately one-third in SCD mice) and transfer it to the endothelium, where heme immediately causes TLR4-mediated ROS production and apoptosis.[202,245] Thereby, targeting and activation of endothelial cells by heme might be facilitated and enhanced through the directional transport via MVs. In contrast, in neutrophils, heme induces antiapoptotic signals via MAPK and PI3K/Akt pathway in a ROS-dependent manner, and delays neutrophil death.[211,214] Neutrophils remain longer functionally active with detrimental consequences. Heme-promoted leukocyte adhesion to the endothelium (TLR4-dependent) as well as NET formation (ROS-dependent, TLR4-independent) has been observed in SCD mice.[210,213] NET formation by heme requires prior cytokine (e.g., TNFα) deposition of neutrophils.[213] Activated leukocytes are also capable of TLR4-dependent TF expression, which promotes the extrinsic pathway of the blood coagulation system, and can be directly induced by heme, too.[407–411] Binding of heme to RAGE might mediate TF expression in leukocytes as well.[396] Furthermore, NETs provide negatively charged surface (RNA) that triggers activation of the intrinsic coagulation pathway.[412]

As already found in the complement system, increasing labile heme levels not only influence cells but also involved proteins. Thereby, both the components of the intrinsic and extrinsic pathway are affected. Heme-induced TF expression on cellular components enables extrinsic initiation of coagulation. Conversely, the intrinsic pathway is activated through direct modulation of FXIIa and plasma kallikrein activity by heme, which leads to the activation of FXI.[240] However, direct binding of heme to FXIIa and plasma kallikrein has not been demonstrated so far.

In the common pathway, heme can influence the tenase complex by binding to FVIII and FVIIIa with high affinity (K_D ~12.7 nM and ~1.9 nM, respectively; stoichiometry: 1:~10 FVIII:heme).[413] As a consequence, the procoagulant activity of FVIII(a) is decreased by ~50%, which is due to impaired FIXa binding.[413,414]

Table 2: Heme-binding capacity of extracellularly available proteins involved in inflammation, the complement, and the blood coagulation system.

Protein*	Suggested binding site (protein:heme ratio)	Heme-binding affinity	Inhibition (↓) or activation (↑)	Year/ Reference
Inflammation				
IL-36α, β, γ (e.g., ~0.6 pM of IL-36α)	Cys 126 [CP], Tyr 108 (1:2) (IL-36α)	~3-4 μM, ~9-13 μM	↓	2019[234]
MD2 (TLR4-associated)	Trp 23/Tyr 34 (1:1)	- (binding observed)	↑ (TLR4)	2020[225]
TNFα (transmembrane)	Cys 30	- (binding observed)	-	2020[224]
Complement system				
C3 (~5 μM)	1 close to ANA domain, 2 in TED/MG2 interface (1:3?)	n. sat.	↑	2013[208]
Factor H (~3 μM)	- (-)	- (binding observed)	↑	2013[208]
Factor B (~2 μM)	- (-)	- (binding observed)	-	2013[208]
C1q (~155 nM)	- (-)	- (binding observed)	↓	2007[242]
	Tyr 122 (A-chain), Trp 190 (C-chain) (1 gC1q :2 heme)	~1-2 μM	↓	2011[241]
	- (-)	~21 nM	-	2020[40]
Blood coagulation system				
Fibrinogen (~9 μM)	Tyr 377 (1:1)	~3.3 μM	↑	2018[21]
FVIII (~0.3 nM)	- (1:~10)	~1.9 nM (FVIIIa) ~12.7 nM (FVIII)	↓	2012[413]
CLEC2 (receptor)	- (-)	~200 nM	↑	2020[18]
RAGE (receptor)	in V-domain (1:~2-3)	~6.8 μM	↑	2020[396]

*Proteins are depicted in plasma concentration-dependent hierarchy and sorted by the involvement in either inflammation, the complement system or the blood coagulation system. A serum/plasma concentration of ~62 mg/l (C1q)[417], ~1 g/l (C3)[418], ~200 mg/l (factor B)[419], ~500 mg/l (factor H)[420] and ~10 pg/ml[421] (IL-36α) is assumed. References for the plasma levels of the proteins of the blood coagulation system are found within Chapter 2.4.1. ANA: anaphylatoxin domain, gC1q: heterotrimeric globular head domain of human C1q, MG2: macroglobulin domain 2, TED: thiol ester-containing domain.

Furthermore, heme binding to the FVIII-VWF complex abolishes the dissociation of the same and leads to the recruitment of the complex to activated platelets.[415] VWF protects FVIII from heme-driven inactivation. In addition, activation of FVIII by thrombin was still possible in the presence of heme.[413] The procoagulant role of FVa, the cofactor in the prothrombinase complex, has been shown to be reduced (by ~80%) in the presence of heme as well.[404] Although thrombin is the main mediator within the plasmatic coagulation, a

potential interaction with heme has not been extensively studied yet. There are only single reports that suggest an inhibition of thrombin by heme.[404,414,416] So far, the actual physiological relevance of these interactions is not understood, since it behaves contrary to the overall procoagulant effects of heme. In contrast, the interaction of fibrinogen with heme and its procoagulant consequence is well-known. While fibrin itself is not affected by heme,[404] fibrinogen binds heme with moderate affinity (K_D ~3.3 μM).[20,21,414,422] The area around Tyr 377 has been suggested as a potential heme-binding site (experimental prove missing). As a consequence, the fibrinogen-heme complex is able to bind onto platelets.[401] Furthermore, heme promotes conformational changes and crosslinking of fibrinogen via dityrosine formation, which might lead to thrombus stabilization.[20,422] Complex formation increased the peroxidase-like activity of heme as well.[20]

Beyond the effects on clotting factors, heme-mediated inhibition of clot lysis was reported, which correlated with the inhibition of plasmin upon heme exposure *in vitro* and further supports the role of heme as an procoagulant and possibly antifibrinolytic alarmin.[414]

Finally, there are only a few reports on direct heme binding to proteins of the blood coagulation with incomplete characterization of the proposed interactions (Table 2). Consequently, there is further demand for the analysis of regulatory heme-protein interactions within the blood coagulation system to characterize the role of heme in hemolysis-driven thrombosis.

2.5 Molecular basis of transient heme binding to proteins and the consequences thereof

In the present thesis, heme binding to extracellular plasma proteins (i.e., of the blood coagulation system) is of particular interest (Table 2, cf. Chapter 2.3 and 2.4.3). These interactions display the epitome of the nature of regulatory heme binding. Regulatory heme binding occurs in a transient fashion leading to the modulation of proteins' activity and/or stability.[423,424] Thereby, surface-exposed short amino acid stretches within the proteins, termed *heme-regulatory motifs* (HRMs), allow for transient heme binding as characterized by both rapid association and dissociation.[423,425] Through the transient nature of regulatory heme binding, quick reactions in acute situations are enabled as can be exemplified by the

already described toxic effects of labile heme under conditions of hemolysis and the consequences thereof (cf. Chapter 2.3 and 2.4.3).

HRMs need to fulfill certain requirements concerning the molecular basis of heme association to protein sequences that were analyzed in detail in recent years.[423,426–431] A fundamental precondition is the presence of an amino acid residue that can serve as a heme-coordination site. Due to the electron-donating side chain of cysteine, histidine and tyrosine, these amino acids display the most common ligands for heme binding.[426,432] The most prominent HRM contains a central Cys-Pro dipeptide (*CP motif*), which has been described for several human heme-regulated proteins in the past (e.g., δ-aminolevulinate synthase 1/2 (ALAS1/2; K_D not known)[425], Bach1 (K_D ~140 nM)[433], and the period circadian protein homolog 2 (Per2; K_D ~12.31 nM)[434]). Since it has been shown that the presence of a CP within the motif does not necessarily account for heme binding, the importance of the surrounding residues has been proposed[432] and from 2011 on extensively investigated.[423,426,428–431,435] Nonapeptides with the consensus sequence X_4(C/H/Y)X_4 were used as models for HRMs, since small peptides were earlier shown to be suitable for studying heme binding.[426,436,437] Due to a high prevalence for additional coordination sites, which evolved in a combinatorial library approach, HRMs were classified (class I-VIII) based on the available coordinating residues (Figure 7a).[426,429,430] The heme-binding capacity of the peptides were analyzed and characterized by a variety of spectroscopic methods, encompassing ultraviolet-visible (UV/vis), resonance Raman (rRaman), continuous wave electron paramagnetic resonance (*cw*EPR) and nuclear magnetic resonance (NMR) spectroscopy.[426–430] Due to the delocalized π-electron system between the central iron(II/III) ion and the macrocyclic porphyrin ring, heme has a characteristic absorbance spectrum with the conspicuous absorbance maximum (*Soret band*) at ~400 nm.[438,439] Thus, changes of the surrounding, as for example induced by binding to a peptide or protein, cause a shift of the Soret band, which is used for respective UV/vis binding studies. Studies with rRaman and cwEPR spectroscopy allowed for the determination of the coordination state of the heme iron ion, whereas NMR provided direct structural analysis of the heme-peptide complexes. In this way, more than 200 nonapeptides (class I-VIII) were analyzed for their heme-binding capacity (i.e., heme-binding mode, ratio and affinity).[423] Motifs of the Cys-based classes I and II formed penta- and hexacoordinated complexes with heme with a Soret band shift to ~420 nm, whereas heme binding to CP motif-based peptides (class III and IV) occurred in pentacoordination with a Soret band shift to ~370 nm.[423,427] For heme-regulated proteins, motifs with a single cysteine (e.g., arginyl-tRNA-synthetase; K_D ~8.6 μM[440]) as well as the CXXCH motif (e.g., Slo1 BK channel; K_D ~0.21 μM (reduced form) and ~2.8 μM (oxidized form)[441,442]) have been reported (despite the CP motif, cf. above). Heme binding to His- and Tyr-based nonapeptides primarily resulted in penta-, hexa- or mixed coordinated states.[430,435] Hexacoordinated heme-peptide complexes were demonstrated to occur either in a sandwich- (two peptide molecules bind

to one heme molecule) or a loop- (one peptide molecule with two coordination sites bind to one heme molecule) like formation, while for the latter at least two spacer amino acids are required.[423,426,427,429,430,435] There are some heme-regulated proteins known that bind heme via a single tyrosine (e.g., progesterone-receptor membrane component 1 (PGRMC1), K_D ~50 nM)[443] or histidine (e.g., Rev-erbβ; K_D ~23 nM (reduced form) and ~117 nM (oxidized form)[444]). Although examples are still rare, these proteins exert comparable high heme-binding affinities as proteins with a CP motif (cf. above). Recently, heme binding via a His-Pro motif (termed *HP motif*) to the mitochondrial heme transporter feline leukemia virus subgroup C receptor-related protein 2 (FLVCR2; K_D ~1 μM) has been shown.[445] So far, this motif did not emerge as an alone standing motif within classes V and VI.[430,435] On the contrary, a detailed analysis of motifs with a combination of histidine and tyrosine residues revealed that in particular motifs with one or two spacer residue are favorable for heme binding.[435] Furthermore, HXH and HXXXH motifs appeared to support an increase of the peroxidase-like activity of heme, which might be helpful for the evaluation of heme's catalytic effect upon binding to proteins as earlier observed for amyloid beta or fibrinogen.[21,446] Thus, beside the well-known CP motif, also combined His-Tyr motifs (especially $HX_{1/3}$(H/Y)-based motifs) might play a superior and so far unexplored/underestimated role in heme binding to proteins.[435] Overall, for all HRMs it is applicable that hydrophobic and aromatic residues support heme binding through an interaction with the macrocyclic porphyrin ring of heme.[426,427,429,430,435] In addition, a positive net charge is favorable and HRMs should be accessible for heme binding within a protein.[426,427,429,430,435]

In the past, protein data bases (such as ScanProsite) were manually screened for potentially heme-regulated proteins based on the requirements for HRMs. As a consequence, various proteins of bacterial (e.g., hemolysin C (HlyC)[447]) and human (e.g., dipeptidyl peptidase (DPP8)[426,427], IL-36α[234]) origin emerged. Upon screening for possible HRMs and subsequent analysis of the protein-derived peptides, heme binding on the protein level was successfully validated and characterized. Subsequently, predicted HRMs were confirmed as relevant heme-binding site by mutational and computational approaches.[234,423,447] Finally, regulation by heme binding has been shown in functional analyses of several proteins upon heme complexation. As such, the enzymatic activity of DPP8 and HlyC, yet also the IL-36α signaling were inhibited in the presence of heme.[234,427,447] However, regulatory heme binding does not necessarily account for an inhibition of the affected proteins. Per2, for example, is degraded upon heme binding, whereas the DiGeorge critical region 8 (DGCR8) and PGRMC1 are activated by heme through the induction of dimerization.[434,443,448] Furthermore, heme facilitates the phosphorylation of the protooncogene tyrosine-protein kinase Src as well as of the Janus kinase 2, which is involved in erythropoiesis.[449,450] Thus, heme is involved in several biochemical processes, such as transcription, circadian rhythm, ion

transport and signal transduction,[424] emphasizing the importance for further investigation of so far unknown heme-regulated proteins.

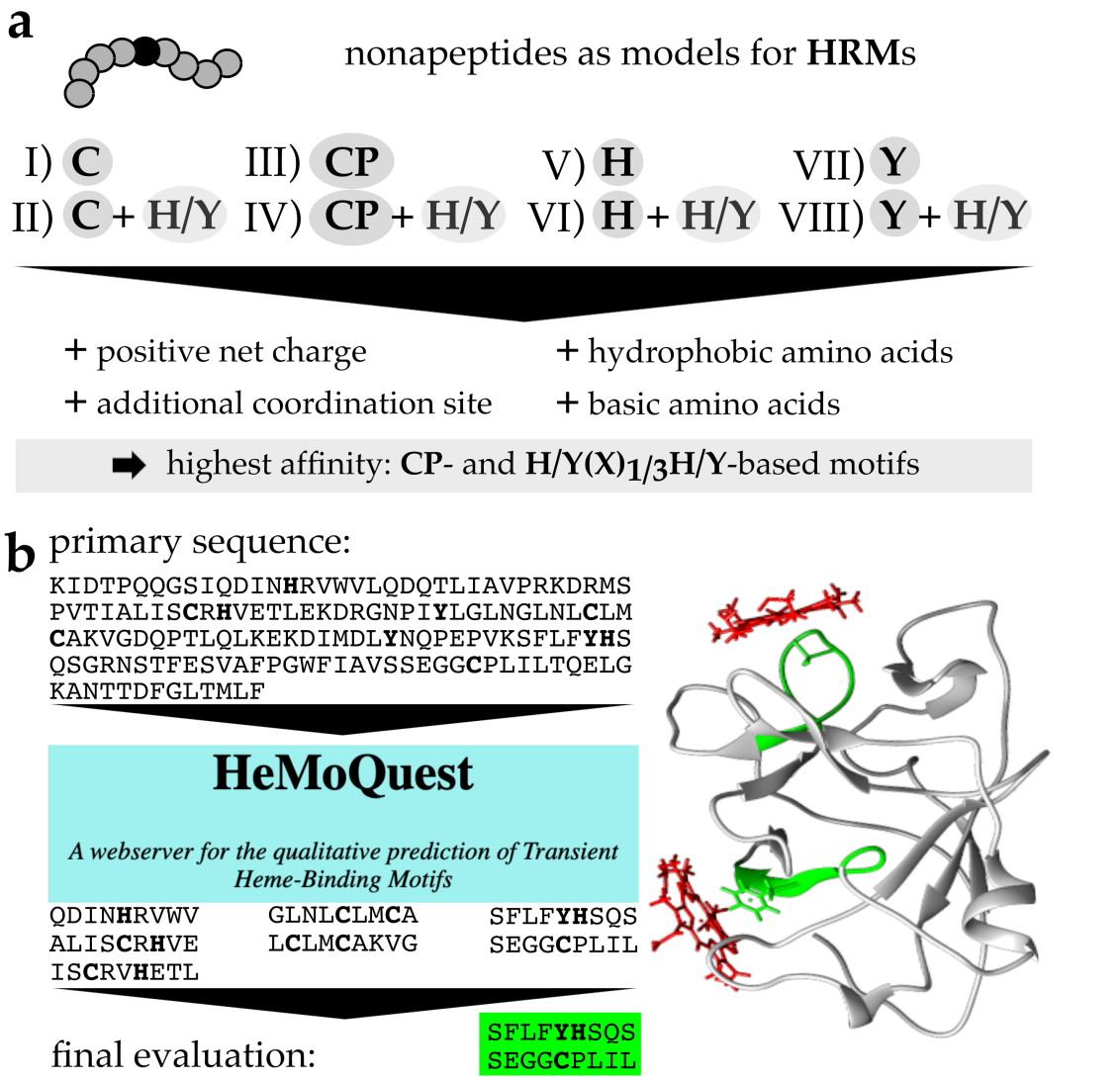

Figure 7: Evaluation of potential HRMs starting from a primary sequence of a protein. a) Nonapeptides were earlier shown to be suitable models for studying the heme-binding capacity of potential HRMs. Based on their properties and the presence of potentially coordinating residues they were subdivided in eight classes. Several studies identified certain favorable requirements for heme binding to these short stretches, including a positive net charge, additional potential coordination sites, hydrophobic and basic amino acids.[423,426–430,435] The most attractive motifs for heme binding are the commonly known CP-motif as well as the recently described H/Y(X)$_{1/3}$H/Y-based motifs. **b)** These criteria were incorporated into the application HeMoQuest, which allows now for automatic HRM screening, starting from primary sequences of proteins.[423,431] As illustrated here by applying the algorithm to the already known heme-binding protein IL-36α[234], HeMoQuest selects potential HRMs from the primary sequence, which need to be finally evaluated by excluding buried sequences (e.g., by using the integrated WESA tool[451]) and motifs involved in disulfide bonds or glycosylation sites (manually). Subsequently, experimental validation (e.g., NMR (right)[234]) is required to confirm the obtained potential HRMs (green).

In order to allow other researchers to evaluate the possibility for transient heme to a protein of interest and predict potential HRMs, the information of experimental data were incorporated into an algorithm (*SeqD-HBM*), which is now publicly available as the webserver *HeMoQuest* (http://131.220.139.55/SeqDHBM/).[423,431] After upload of the primary sequence of a protein, potential HRMs are listed based on the already described, experimentally gained criteria. Furthermore, if desired, the implemented WESA tool allows for further refinement of the selection due to automated exclusion of non-exposed motifs.[431,451] In the end, only those motifs need to be manually dismissed that are involved in specific modifications, such as disulfide bonds or glycosylation sites (Figure 7b). Just recently, the application was used for the prediction of heme-binding sites in sideroflexin (SFXN) 1, which has been suggested to play a role in iron homeostasis.[452] Furthermore, proteins of the blood coagulation system were screened for their heme-binding ability, which revealed potential candidates, such as the blood coagulation inhibitor APC (cf. Chapter 4.2), and laid the basis for the present thesis.

3 Thesis outline

This thesis aims to contribute to the understanding of the molecular basis of the thrombophilic state of patients with hemolytic disorders. As labile heme is released in large amounts under hemolytic conditions, it has been suggested as a critical factor in hemolysis-driven hypercoagulability.

Thus, within the first part of the present thesis (cf. **Chapter 4.1**), approximately 110 years of research on heme as an actor in the blood coagulation system are summarized. This review presents heme as a versatile operating molecule targeting participating cells (e.g., platelets and endothelial cells) as well as proteins (e.g., FVIII and fibrinogen). However, although there are several consequences of the prothrombotic role of heme already described, it emphasizes the deficit of knowledge on the molecular level and consequently, the need for further investigations.

To address this objective, the second part (cf. **Chapter 4.2**) comprises the identification and extensive analysis of the anticoagulant serine protease APC as a so far unknown heme-regulated protein. Based on earlier established criteria and with the help of the webserver HeMoQuest,[423,426–431] potential HRMs were identified and analyzed for their heme-binding capacity. Furthermore, functional investigations with biochemical and clinical assays, which were partially performed in cooperation with Dr. N. S. Hamedani and Prof. Dr. B. Pötzsch (University hospital Bonn), provide valuable insights into another way of a procoagulant action of heme on the molecular basis through the inhibition of an anticoagulant enzyme.

In order to further evaluate the interrelations in heme-driven effects under hemolytic conditions, the third part (cf. **Chapter 4.3**) presents a first compilation of heme's large radius of action under hemolytic conditions (in cooperation with Dr. D. Domingo-Fernández and Prof. Dr. M. Hofmann-Apitius (Fraunhofer SCAI)). In the computationally generated network, called heme knowledge graph (*HemeKG*), in particular proinflammatory and prothrombotic pathways emerged. HemeKG allows for the prediction of interrelations on the molecular basis, which can support further experimental analysis in the future.

The fourth part (cf. **Chapter 4.4**) demonstrates the application of HemeKG for the analysis of potential heme-mediated effects in pathological states as herein exemplified on the coronavirus disease of 2019 (COVID-19). Upon comparison of HemeKG and a knowledge graph of COVID-19[453] (in cooperation with Dr. D. Domingo-Fernández and Prof.

Dr. M. Hofmann-Apitius (Fraunhofer SCAI)), several parallels between heme-driven pathologies and COVID-19 pathology were identified, as shown by common biomarkers of inflammation, the complement, and the blood coagulation system. In addition, potential heme-binding proteins were found that might be regulated by heme. Both approaches recommend the consideration of preexisting hemolytic disorders in COVID-19 that might trigger an even worse disease progression.

In the last part of this thesis (cf. **Chapter 5**), a final conclusion follows including the main findings of this work.

4 Manuscripts

4.1 Linking labile heme with thrombosis

Review article

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4.1.1 Introduction

The massive release of labile heme into the vascular compartment has been associated with cytotoxic, proinflammatory, and prooxidant effects, thereby characterizing the pathogenesis of hemolytic disorders, such as sickle cell disease, thalassemia and paroxysmal nocturnal hemoglobinuria.[6–11] The thrombophilic state of patients with hemolytic attacks was attributed to heme as well.[13,14,395] So far, the underlying mechanisms are not entirely known. This review article collects the current knowledge of heme as a modulator within the blood coagulation system and its role in the development of thrombotic complications under hemolytic conditions. Furthermore, coagulation abnormalities of acute intermittent porphyria patients upon heme infusion are described. In particular, interactions of heme on the molecular and cellular level are highlighted.

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Review

Linking Labile Heme with Thrombosis

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Abstract: Thrombosis is one of the leading causes of death worldwide. As such, it also occurs as one of the major complications in hemolytic diseases, like hemolytic uremic syndrome, hemorrhage and sickle cell disease. Under these conditions, red blood cell lysis finally leads to the release of large amounts of labile heme into the vascular compartment. This, in turn, can trigger oxidative stress and proinflammatory reactions. Moreover, the heme-induced activation of the blood coagulation system was suggested as a mechanism for the initiation of thrombotic events under hemolytic conditions. Studies of heme infusion and subsequent thrombotic reactions support this assumption. Furthermore, several direct effects of heme on different cellular and protein components of the blood coagulation system were reported. However, these effects are controversially discussed or not yet fully understood. This review summarizes the existing reports on heme and its interference in coagulation processes, emphasizing the relevance of considering heme in the context of the treatment of thrombosis in patients with hemolytic disorders.

Keywords: blood coagulation; coagulation factors; heme binding; hemolysis; hemolytic diseases; hemorrhage; labile heme; platelets; thrombosis

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1. Introduction

Worldwide, one in four people die from cardiovascular diseases related to thrombosis [1]. In the case of thrombosis, an imbalance of blood coagulation occurs, leading to the formation of a blood clot, and consequently to partial or complete vascular occlusion [2]. Whether arterial or venous, a blockage in the vasculature causes a limitation of the blood flow, which usually relies on a complex homeostatic interplay of plasma proteins (i.e., coagulation and inflammatory factors), blood cells (in particular platelets and red blood cells (RBCs)) and the endothelium [2,3]. While arterial thrombosis, such as in atherosclerosis, commonly follows the rupture of an atheroma under conditions of high shear stress, venous thrombosis, such as deep vein thrombosis (DVT), occurs mostly at sites of intact endothelium under low shear stress conditions [4–6]. Apart from inherited coagulation disorders, major risk factors for thrombotic abnormalities are for example age, life-style factors, such as smoking and obesity, as well as trauma and surgeries [7–10]. As such, thrombotic events contribute to a variety of clinical sequelae encompassing ischemic heart disease and stroke [2,6,11]. Moreover, several hemolytic conditions, such as sickle cell disease (SCD) [12–24], hemolytic uremic syndrome [25,26], hemoglobinuria [27,28], hemolytic transfusion reactions [29–36], hemorrhage [33,37–42], and cardiac surgery [43,44], were reported to manifest thrombotic complications as thrombosis, hypercoagulability, and vasculopathy [45–56]. In adult SCD patients, venous thromboembolism (VTE) is even one of the leading causes of death [57,58]. The thrombophilic status of patients is likely driven by multiple factors. Therefore, the following changes in hemostatic biomarkers are striking: Elevated levels and/or activity of clotting proteins (e.g., tissue factor (TF) [59,60], factor VIII (FVIII) [45,61,62], fibrinogen [45]), reduced levels of contact proteins (e.g., factor XII (FXII) [63], prekallikrein [63,64], high molecular weight kininogen [63,65]), increased levels of fibrinolytic markers (e.g., D-dimers [28,66,67],

fibrinopeptide A [68,69], plasmin-antiplasmin complexes [16,67], fibrinogen-fibrin degradation products (FDP) [70,71]), increased expression of adhesion proteins (e.g., intercellular adhesion molecule 1 (ICAM-1) [72,73], vascular cell adhesion molecule 1 (VCAM-1) [72,74], von Willebrand factor (VWF) [75,76], P-selectin [28,74,77], thrombomodulin [78,79]), decreased levels and/or activity of anticoagulant proteins (e.g., protein C [68,80–83], protein S [68,80–82], antithrombin [84]), and an increase of platelet activation [85,86]. In addition, exposure of negatively charged phosphatidylserine on cell surfaces is increased providing a binding site for accumulated enzyme complexes, which further enhances procoagulant activity through support of thrombin generation and platelet activation [19,87,88]. However, the concrete underlying mechanism is still not entirely known [48,50].

These hemolytic disorders are associated with an excessive release of labile heme, which itself is capable of exerting a great variety of functions [89–92]. As a permanently bound prosthetic group, heme equips numerous hemoproteins with their different properties [92,93]. The vital role of heme in the cardiovascular system can be demonstrated using the examples of oxygen transport by hemoglobin, antioxidant activity of peroxidases and catalases or electron transfer by cytochromes [92–94]. Moreover, heme is well-known as a signaling molecule that acts through transient binding to various proteins mediating a wide range of biochemical processes, such as transcription, inflammation, or signal transduction [90–92].

In the human body, 80% of heme is produced and found in RBCs, of which there are up to 25 trillion occurring in the circulation [90,95]. Therefore, the concentration of extracellular heme is relatively low under physiological conditions [92,96]. In hemolytic situations, extracellular labile heme levels can reach dangerously high concentrations [97,98]. While extravascular hemolysis results in the phagocytosis of RBCs by macrophages of the reticuloendothelial system (RES) especially in the liver and the spleen, intravascular hemolysis leads to the lysis of RBCs and release of the RBC content into the vascular compartment [99–101]. The damage of each RBC can result in the release of about 2.5×10^8 hemoglobin molecules from a single RBC into the bloodstream [102]. After dissociation into $\alpha\beta$ dimers, free hemoglobin is scavenged by the acute phase serum protein haptoglobin, thereby preventing from hemoglobin-mediated oxidative damage, renal infiltration, and iron loading [96,103–106]. Subsequently, the complex is recognized by the macrophage-specific surface protein cluster of differentiation (CD) 163, which arranges the uptake into macrophages of the RES and, thus, the clearance of both, hemoglobin and haptoglobin, from circulation by lysosomal breakdown to heme, peptides and amino acids [107–110]. The resulting heme is sequestered by the heme oxygenase system yielding biliverdin, iron ions, and carbon monoxide [107,111,112]. Heme oxygenase 1 (HO-1) expression can be induced by heme itself, which consequently prevents from harmful effects like oxidative stress, inflammation or ischemia-reperfusion injury [113,114]. However, in case of more extensive hemolysis, the hemoglobin-binding capacity of haptoglobin becomes overwhelmed, followed by the accumulation of hemoglobin in plasma [107,114]. Hemoglobin is then oxidized to methemoglobin [115,116], which results in the release of large amounts of labile heme [117–119]. Consequently, heme is scavenged by different plasma proteins, in particular hemopexin, α1-microglobulin, albumin, and lipoproteins, and eventually detoxified through the formation of non-toxic heme-protein complexes [96,99,113]. First, liberated heme is mainly bound to albumin as the most abundant protein in plasma (~300 μM) possessing a low affinity (K_D ~40 μM) and a high affinity binding site (K_D ~20 nM) for heme [120,121]. Subsequently, it is transferred to hemopexin, which is known as the heme-binding protein with the highest heme-binding affinity (K_D ~5.3 fM) [122,123]. The plasma level of hemopexin (~20 μM) is considerably lower than of that of albumin, explaining the initial association of heme with albumin [110]. Hemopexin then transports heme to the parenchymal liver cells, where the internalization of the heme-hemopexin complex is directed by the low-density lipoprotein receptor-related protein 1 (LRP1)/CD91, which results in the cellular uptake and degradation of heme [99,124]. Nevertheless, in severe hemolytic states, such as occurring in sickle cell disease, ischemia-reperfusion injury or blood transfusion [125–127], these

heme detoxification systems become overwhelmed. In this case, excess of heme can lead to toxic effects, evoking oxidative stress, inflammation and hemolysis [94,128,129]. Due to the redox-active nature of the iron ion in labile heme, its toxicity is mainly based on the heme-induced generation of reactive oxygen species (ROS), leading to damage of lipid membranes, nucleic acids, and proteins by Fenton reaction and subsequently to cellular injury and cell death [113,130–132]. In addition, heme-mediated ROS production and membrane damage of RBCs can induce hemolysis, which, in turn, leads to a further increase of extracellular heme levels [113,131]. Heme has also been recognized as a damage/danger-associated molecular pattern (DAMP) triggering a large number of proinflammatory pathways (e.g., nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), and specificity protein 1 (SP-1) signaling) [129,133].

Therefore, it is not surprising that patients with any diseases accompanied by hemolysis suffer from heme toxicity related symptoms, in particular acute proinflammatory responses [98,134–136]. A role of heme in the induction and mediation of the thrombotic effects accompanying hemolysis is discussed as well [97,98,137]. Here, we review the role of heme and its interactions with components of the blood coagulation system and discuss the implications of heme in initiating and processing thrombosis of patients with hemolytic diseases.

2. Thrombotic Complications upon Heme Injection

First trials to study the effect of hematin (ferri(Fe^{3+})heme hydroxide) injection can be traced back to the year 1911 [138], shortly before the structure elucidation of hemin (ferriheme chloride) was completed by W. Küster (1912) [139]. Actually, the study aimed at deciphering the origin of hematosiderin (an iron-storing complex) and bilirubin ("hematoidin") from hematin as a potential source and suggested, for the first time, its release from degraded hemoglobin from ruptured RBCs [138]. Experiments with intravenous and intraperitoneal injection of an alkaline hematin solution (30–54 mg/kg) in guinea pigs and rabbits, respectively, had to be abandoned due to the extreme toxicity of the solution. An autopsy after subcutaneous and intraperitoneal injection of hematin and hemin, both suspended in salt solution at a concentration of 30 mg/kg produced hemorrhage and ecchymosis, an extravasating bleeding usually associated with small vessel lesions in subcutaneous tissues [138]. Moreover, a pronounced tendency towards hemorrhage was observed in the affected and surrounding tissue [138]. In 1913, this was confirmed in cats and dogs after injection of hematin (in the range of 3.5–9 mg per kg body weight) into the viscera and the peritoneal cavity [140], as well as in rabbits, where it occurred as hemorrhagic kidney injury and/or extensive hemorrhage in the peritoneal cavity after injection of large hematin doses (10–25 mg per kg) [141,142]. Furthermore, W. H. Brown described the formation of hyaline thrombi or emboli in the smaller, primarily glomerular vessels and subsequent occurrence of vaso-occlusion and vascular injuries. In some cases, even infarcts were observed [141]. This study thus provided a first indication of heme-induced thrombotic complications (Supplementary Table S1). About 30 years later, Anderson and colleagues injected hematin (i.a., 200 mg intraperitoneally and 20 mg subcutaneously) in dogs and confirmed these observations [143]. They noted, in particular upon intravenous injection, conspicuous changes of the vasculature, comprising congestion, hemorrhage, and thrombosis, especially in small vessels. In addition, the authors stated that the observed effects were highly similar to those usually occurring under hemolytic conditions [143]. In contrast, Corcoran and Page described in 1945, among other symptoms, an anticoagulant effect after hematin injection into dogs, resulting in an inhibition of the coagulation process [144]. In 1966, Gessler and coworkers were faced with massive hemorrhagic bleeding in rats after injection of hematin (100–180 mg per kg body weight). The rats died a few seconds after injection, while bleeding from every possible body orifice [145].

As the aforementioned studies were mostly performed in view of the characterization of the malarial pigment (today known as the crystallized form of heme "hemozoin"), in 1971 researchers started to consider the therapeutic use of heme in the context of

porphyria treatment [146]. Therefore, more experiments for the clarification of heme toxicity were undertaken (Supplementary Table S1) [147]. Hemin dissolved in 1% Na_2CO_3 was intravenously administered in rats (30–60 mg per kg body weight). Thereby, most of the rats (88.9%) that received the lowest dose (30 mg hematin per kg body weight) stayed alive. After injection of 40 mg hematin per kg body weight, for 30% of the rats in the experimental group internal bleeding was discovered postmortem [147]. With the highest dose (60 mg per 1 kg body weight) applied, 100% of the rats died. As previously observed, bleeding into small intestine and petechiae of liver, lungs and adrenals were found. In addition, hemorrhage and subcutaneous hematomas were detected. The LD_{50} was determined to be 43.2 mg hematin per kg body weight [147]. At that time, porphyria patients (acute intermittent porphyria (AIP), porphyria variegate or hereditary coproporphyria) were already successfully treated with hematin [148,149]. The administered dose of 4 mg/kg was effective and reported without any negative side effects, suggesting the relevance of coagulopathies as a consequence of heme injection only upon administration of excessive amounts [147,148]. Therefore, hematin was introduced as a drug (Panhematin®) in the USA in 1983 [150].

In contrast, in 1975 Dhar et al. observed thrombophlebitis after hematin infusion (prepared in alkaline solution, 1.2–6 mg/kg) in patients with hepatic porphyria [151]. Few years later, Lamon et al. reported chemical phlebitis at the site of hematin infusion in some cases, although less hematin was administered (2 mg/kg; reconstituted in saline solution) [152]. Here, the infusion was organized in a consecutive, daily manner, while applying hematin (2 mg/mL in saline solution) over a time period of 15 minutes (min) as the maximum infusion time [152]. In 1981, Morris and coworkers confirmed these observations in a patient with AIP. Here, 196 mg hematin was intravenously injected every 12 hours (h). Afterwards, pronounced coagulation was determined, without an explanation of the underlying mechanism [153]. In the following years, more and more studies supported the procoagulant effect of hematin as evidenced by thrombophlebitis. In the study of McColl et al., hematin was prepared in a 1% Na_2CO_3 solution and stored at 4 °C up to 10 days before use [154]. A dose of 4 mg/kg was intravenously infused in patients with acute porphyria attacks either every 12 h or as a daily dose. As a consequence of the injection in a small peripheral vein, phlebitis arose in up to 30% of the patients, in two cases even severe phlebitis [154]. Sometimes comparable hematin doses (3–4 mg/kg) resulted in the formation of bile thrombi [155]. These thrombophilic reactions were reported not only in patients with porphyria, but also in healthy volunteers (in 45% of the cases) upon heme administration (4 mg/kg body weight) [156]. Thrombophlebitis was limited to the vein in which hematin was infused. In patients without thrombophlebitic reaction, fibrotic events as well as vaso-occlusion were observed [156]. In contrast, hemostatic parameters suggested an anticoagulant role of hematin (Section 3) [156].

Since these observations were not uniform, varying from mild to severe effects, as well as from bleeding to thrombotic events, Goetsch and Bissell suggested the instability of hematin as the major cause for these differences [157]. In 1988, Simionatto et al. correlated hemostatic parameters with the actual hematin concentration in the plasma of nine test persons. They found a 30% loss of hematin in plasma, which might be an evidence for the conversion into related degradation products [156]. In addition, it might be also an indication for the instability of the hematin solutions, conceivably responsible for the thrombotic effects [156]. Due to the instability of hematin there were efforts deployed to improve its effectiveness and reduce adverse effects. In 1987, Tenhunen and coworkers presented for the first time a stable, administrable heme compound, so-called heme arginate (later approved as drug "Normosang®") [158]. The compound was prepared as hemin arginate (corresponding to 25 mg/mL hemin) in an aqueous solution with 40% 1,2-propanediol and 10% ethanol added. Even after repetitive infusion of heme arginate (5 mg/kg) in rabbits there were no side effects observed, in particular no thrombophlebitis or other signs of prothrombotic states. In comparison, the administration of hematin (in form of the drug "Panhematin®") resulted again in thrombophlebitic events [158]. In mice, the LD_{50} for

heme arginate was determined to be 56.3 mg/kg (intravenous administration), 112.5 mg (intraperitoneal administration) and >5 g (oral administration) [158]. Therefore, the LD_{50} of heme arginate is fundamentally higher than for hematin [147,158]. Clinical trials with heme arginate (Normosang®) were already started at that time, forming the basis for the introduction as a drug in the European pharmaceutical market [158]. The higher safety with heme arginate treatment might be due to the fact that it is not as potent as heme in catalyzing free radical reactions and thus sensitizes endothelial cells to oxidant injury to a lesser extent [159,160]. Nevertheless, thrombophlebitic or bleeding effects occasionally appeared in humans as a complication as well (Supplementary Table S1) [161–165]. In another approach, heme complexed with albumin was administered [166]. The compound, consisting of 0.5 mM heme and 0.5 mM albumin, was well-tolerated in all test persons and showed no evidence of instability. Thereby, neither thrombosis nor bleeding was observed, probably because of complexation of heme with albumin [166,167].

In the USA, Panhematin® was and still is the only approved heme-based drug and, thus, the compound of choice. Therefore, physicians are still faced with the abovementioned disturbances of the hemostatic system in the context of heme therapy in porphyrias. In 2000, Gajra and coworkers reported another case of an AIP patient that developed clinically obvious coagulopathy after hematin (Panhematin®) treatment as a single dose of 4 mg/kg [168]. The patient recovered 72 h after treatment. More recently, in the broad study of Anderson and Collins reported in 2006 it was found that 3.1% of the treated patients showed thrombotic complications as side effects after hematin administration [150].

Taken together, there were bleedings and hemorrhages observed, partially postmortem, when animals were treated with immense doses (14–180 mg/kg) of hematin or hemin (Figure 1, Supplementary Table S1). In addition, upon administration of hematin in the range of 10–25 mg/kg, both, hemorrhage and thrombotic complications, occurred. In humans, either healthy or porphyria patients, considerably lower concentrations of hematin or heme arginate were injected, resulting in thrombotic complications. The only exceptions are a report on the infusion of 2–3 mg/kg heme arginate, which led to occasional bleeding in patients with myelodysplastic syndrome [162] as well as the description of coagulopathies and hematomas in an AIP patient who was treated with Panhematin® (Supplementary Table S1) [168].

Based on these observations, authors recommended complete monitoring of coagulation parameters, such as clotting times and platelet count, during hematin therapy in porphyria patients [153,168,169].

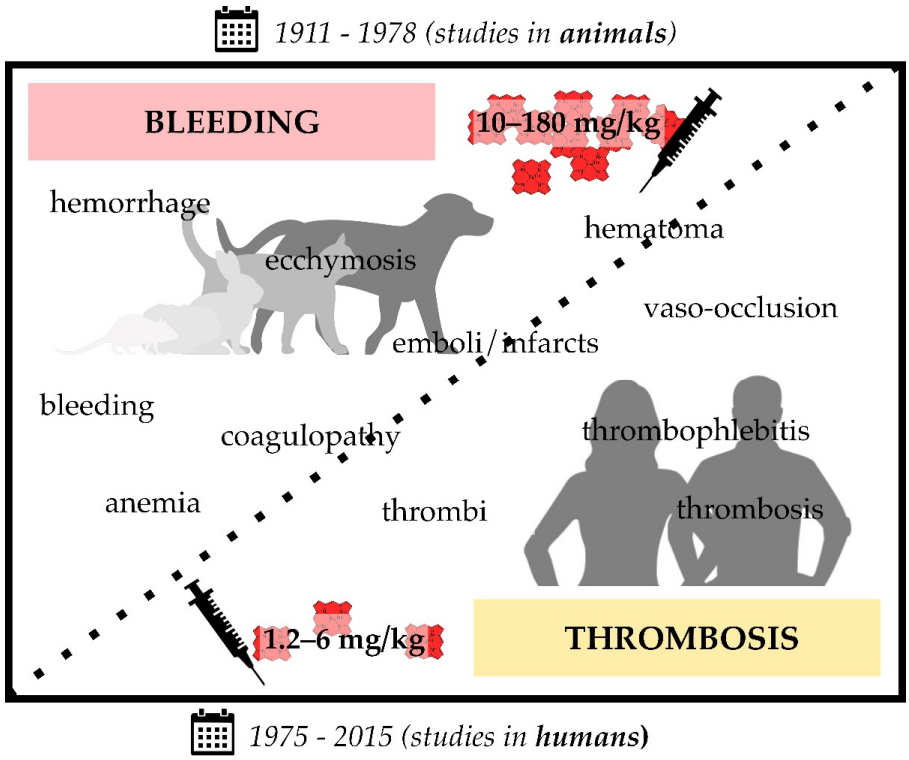

Figure 1. Infusion of heme in different formulations (i.e., hematin, hemin, heme arginate (Normosang®), Panhematin®) leads to coagulation disorders in animals (1911–1978; rats, guinea pigs, rabbits, cats, and dogs) and humans (1975–2015). Heme infusions of rather high concentrations (10–180 mg/kg) were shown to cause bleeding symptoms in animals, such as hemorrhage and coagulopathy, whereas administration of comparatively low concentrations (1.2–6 mg/kg) resulted in prothrombotic symptoms, such as vaso-occlusion and thrombophlebitis. The transition between bleeding and thrombotic events seems to be smooth, since there are also studies that report both effects in the same study (10–25 mg/kg hematin) [141,143]. Moreover, there are single exceptions (three studies) that also report bleeding upon administration of lower hematin (3.5–9 mg/kg) [140,162] or heme arginate (2–3 mg/kg) [168] concentrations.

3. Heme-Mediated Interference in Coagulation Point-of-Care Testing

Since screening of coagulation factor levels is usually time consuming and appropriate tests are of limited availability, various routine coagulation tests are employed conventionally in order to characterize the hemostatic state of patients. Today, the determination of the activated partial thromboplastin time (aPTT) and the prothrombin time (PT) are common procedures. The aPTT is usually used to control the proper course of the intrinsic and common pathway of coagulation. Citrated plasma is mixed with phospholipids and a contact activator, such as Kaolin [170]. Consequently, factor XI is activated, but only when calcium ions are added the coagulation can proceed. The time to complete clotting is then recorded as aPTT. Clotting factor deficiency (i.e., factor (F)I, FII, FV, FVIII, FIX, FXI, FXII, high-molecular-weight kallikrein and kallikrein) or an impaired activity of the same results in an aPTT prolongation [170]. In contrast to the aPTT, PT allows for the evaluation of the extrinsic and common coagulation pathway. Thus, first TF is added to activate the extrinsic pathway, and then calcium ions. From prolonged PT, impaired levels or activity of the following clotting factors can be derived: FI, FII, FV, FVII, and FX [171]. To enable a comparison between the results from different laboratories, sometimes a standardized prothrombin time ratio, the international normalized ratio (INR), is determined [172]. Among aPTT and PT screening assays, other clotting and bleeding times that were analyzed in the past and/or still today are the following: Thrombin time (TT; fibrinogen-dependent), fibrinogen time (FT; fibrinogen-dependent), reptilase time (RT; fibrinogen-dependent), bleeding time (platelet-dependent), and the ethanol gelation test (fibrinogen-dependent). These were also used to characterize the effect of heme in its different formulations on the coagulation process in vivo and in vitro (Supplementary Table S2).

Already in 1913, a prolongation of the bleeding time (Duke method) was observed as a consequence of hematin injection (25 mg/kg) in rabbits [142]. The animals continued bleeding for several hours (>2 h) after a small cut into the ear. In consent with these observations, Brown et al. showed that hematin (25 mg/kg) was capable of prolonging the coagulation time in rabbits from an average value of 8–11 s to 17 s [142]. In the same setting, prolongation of the bleeding time was more pronounced. Indeed, Barnard (1947) observed a similar, concentration-dependent effect on the TT by adding 20–60 mg.% lithium ferriheme to plasma samples, even up to a complete loss of clotting ability (Supplementary Table S2) [173]. The author suggested that this might be due to heme interaction with for example thiol groups that might play a role in blood coagulation. In contrast, lithium ferriheme did not induce any significant changes of TT [173].

About 35 years later, Morris and colleagues treated a female AIP patient with hematin (196 mg, every 12 h) and observed markedly prolonged PT (from 13.2 s to 20.2 s), as determined 11 h after the first hematin administration [153]. The same was realized for the aPTT (from 25–41 s to more than 60 s), but no sign of thrombophlebitis occurred [153]. Upon hematin infusion (4 mg/kg, every 12 h) in an AIP patient, Glueck et al. confirmed the previous observations with both, an increase of the PT (from 11.7 s to 18.3 s) and a marked prolongation of the aPTT (from 37.5 s to more than 150 s) [169]. In contrast, the TT stayed unaltered. Based on these observations, the group continued with in vivo studies, focusing on the effect of hematin on the PT, aPTT, TT, and RT. For this purpose, 4 mg/kg hematin was administered by intravenous infusion over a period of 15 min, blood samples were taken over a time range of 0–48 h after infusion and subsequently processed for hemostastic characterization by clotting times. Already 10 min after infusion all parameters were extended in the collected samples: The aPTT increased from ~30 s to ~100 s, the PT from ~4 s to ~10 s, the TT from ~10 s to ~25 s, and the RT from 20 s to 30 s [169]. In a second approach, the same patient was pretreated either with 650 mg acetylsalicylic acid or with 5000 U heparin 2 h and 10 min, respectively, before hematin infusion (4 mg/kg). Interestingly, although acetylsalicylic acid and heparin are already potent anticoagulant compounds, the addition of hematin still increased the effect on the clotting times in comparison to the acetylsalicylic acid and heparin baseline [169]. However, in all approaches the coagulation parameters returned to normal levels (latest 48 h after hematin injection), with hematin levels also normalized [169]. In vitro, hematin (0.01 mg/mL and 0.1 mg/mL, respectively) prolonged the TT and RT of normal plasma as well. In the same year, Green et al. confirmed that hematin (0.01 mg/mL) is capable of prolonging the TT (from ~13 s to ~46 s) [174]. Upon administration of hematin in complex with albumin a fourfold concentration of hematin was necessary to induce a prolongation of the TT [174]. In contrast, Morris et al. did not detect bleeding in an AIP patient after hematin treatment (196 mg, every 12 h) [153].

Shortly thereafter, it became doubtful whether or not the observed effects were induced by hematin itself [156,157,175]. Goetsch and Bissell (1986) compared the PT of plasma in the presence of fresh and aged hematin (40 mg/L) in vitro [157]. While freshly prepared hematin (in 0.1 M Na_2CO_3, pH 8.0) did not affect the PT, stored hematin slightly prolonged this clotting time in vitro. This effect was more pronounced when hematin was stored longer and at room temperature (e.g., after 50 h of storage: ~16.3 s) instead of storage at 4 °C (e.g., after 50 h storage: ~15.8 s). Based on these results the authors recommended the use of freshly prepared hematin solution for infusion in the treatment of AIP, in order to prevent coagulopathies as a side effect of hematin administration [157]. In the course of the detailed study of Simionatto et al. (1988) on thrombophlebitis following hematin administration (4 mg/kg), clotting times in nine healthy volunteers were monitored [156]. At the time of maximal plasma heme levels (~50 µg/mL), a prolongation of aPTT (+~24%), PT (+~20%) and TT (+~13%) was observed. The normal range was reached after 9 h (for aPTT), 24 h (for PTT), and 7 h (for TT) after hematin injection. For aPTT, the results were confirmed in vitro, when hematin (70 µg/mL) was added to untreated plasma (+~31%) [156]. Simultaneously, R. L. Jones (1986) hypothesized that in aged (up to 50 days, 10 mg/mL) hematin solution oxidatively degraded products are responsible for the anticoagulant effects [175]. In their

approach, freshly prepared hematin solution did not cause any change of the clotting times (PT, TT, aPTT), whereas aged hematin solutions greatly prolonged the clotting times up to ~2.8-fold (aPTT, 50 days old solution, phosphate buffer, final concentration: 60 µg/mL) (Supplementary Table S2). Thus, the author determined the actual hematin concentration within the solutions by using the pyridine hemochromogen assay, revealing a 50% reduced actual hematin concentration. Indeed, chromatographic analysis (thin-layer and high-performance liquid chromatography) revealed a different pattern between aged and freshly prepared hematin solution. However, to date, structural elucidation and validation of these degradation products with anticoagulant function is missing. In order to prove the assumption of oxidative degradation products, R. L. Jones added antioxidant and iron-chelating compounds, which, indeed, were able to suppress the anticoagulant effect of the aged hematin solutions [175]. Furthermore, fast in vivo generation of the anticoagulant hematin degradation product(s) was suggested, which was derived from experiments in rats that received infusion of hematin (12 mg/kg) with a parallel monitoring of the plasma hematin concentration [175]. Finally, the author also compared the effect of the freshly prepared hematin solutions on the clotting times with that of Panhematin®. In contrast to freshly prepared hematin solutions, in case of Panhematin® that was freshly reconstituted in water (as by instruction), a significant prolongation of the clotting times was recorded [175]. Due to these studies concerning the anticoagulant effect of aged hematin and Panhematin® [157,175], Simionatto and colleagues spectroscopically examined the constitution of their hematin solution (Panhematin®). They detected changes in the absorbance spectra indicating the degradation of hematin. Thus, again, the anticoagulant effect was assigned to the potential degradation products of hematin by the authors [156].

In contrast to all other studies, Becker et al. (1985) observed a shortened partial thromboplastin time (PTT; by ~30%) induced by hematin (3 nmol) in vitro, which was interpreted by the authors as an hematin-mediated activation of the intrinsic blood coagulation cascade [176]. However, they also found an increase in fibrinolysis after hematin addition as determined by a pronounced decrease (by ~78%) of the euglobulin clot lysis time.

At the same time, clotting parameters in the presence of heme arginate were investigated in healthy volunteers (Supplementary Table S2) [158,177]. Tenhunen and coworkers injected heme arginate at a dose of 3 mg heme per kg and monitored coagulation parameters before and 15 to 240 min after injection. Consistent with the clinical trials (Section 2), no changes of the results obtained from the various hemostatic point-of-care tests (aPTT, PT (quick time), TT and ethanol gelation) were observed [158]. The same group repeated these experiments and correlated the results with the actual maximal plasma heme concentrations [177]. The latter was reached about 30 min after injection and determined to be ~51.5 µg/mL with a half-life of about 11 h. Again, the clotting times remained in the reference range and no significant changes were observed in the presence of heme (3 mg/kg) administered as heme arginate [177]. Unfortunately, the authors did not record these parameters at the time of the maximal plasma heme concentration. In 1989, Herrick et al. also monitored the hemostatic parameters of AIP patients that were treated with 3 mg/kg heme arginate. In most of the patients, heme arginate did not affect PT and aPTT. Only one out of 12 patients showed a prolonged aPTT, but upon both placebo and heme arginate treatment, suggesting an already preexisting coagulation disorder without any correlation with heme infusion [165].

Since 1990, further case reports of AIP patients, who were treated with Panhematin®, were published (Supplementary Table S2) [168,178]. For example, Gajra and colleagues intravenously infused 4 mg/kg of the hematin drug and monitored prolonged aPTT (~1.6-fold), FT (~1.2-fold), INR (from 1.19 to 1.52), and TT (~1.4-fold) 5 h after the treatment, which supported their clinical observations (Section 2) [168]. In contrast, Green and Ts'ao only observed a marginal increase of aPTT and PT after the second infusion of hematin, whereas the TT remained unaltered [178]. So far, there is no explanation for the differing results of both studies [168,178].

In 2003, Huang et al. tried a similar approach as Green et al. [174] using heme in complex with albumin and analyzing its effect on clotting times, but with the difference that Huang et al. aimed at the characterization of the heme-albumin complex as a new RBC substitute through its ability to carry oxygen [174,179]. Therefore, whole blood of rats was mixed with a recombinant human serum albumin (50 g/L)—heme (5 mM) solution (aqueous, 0.9% NaCl, pH 7.4). The analysis revealed no significant changes of the aPTT as well as of the PT [179].

Further projects followed that focused on the clarification of the effect of hemin on hemostatic parameters (Supplementary Table S2). The study of Rochefort et al. from 2007 supported the earlier results concerning a potential anticoagulant effect of heme with a high-frequency ultrasound technique [180]. The acoustic velocity of whole blood in rats, which underwent daily hemin treatment (50 mg/kg), was analyzed at 500 MHz. Upon hemin administration clotting velocity was significantly decreased up to a degree comparable to heparin treatment (500 IU/kg, daily). Whole blood from hemin-treated rats needed approximately 40 min to clot formation, while blood from control rats clotted much faster (within ~10 min). Furthermore, the effect was characterized by a decreased slope of the increase in acoustic velocity (0.005 m/s^2 (hemin-treated) vs. 0.031 m/s^2 (control)), and a lower final velocity (12 m/s (heme treated) vs. 30 m/s (control) after 100 min) [180]. The same group intraperitoneally injected pure hemin (50 mg/kg) in male Wistar rats, with hemin dissolved in 0.5% DMSO before administration. No significant change of the PT and aPTT was monitored here [181]. In the same year, Desbuards et al. described a preventive role of hemin for thrombosis. In the study, carotid thrombus formation in rats was induced by electrical stimulation [181]. The thrombus induced in all control rats accumulated RBCs and dystrophic endothelial cells, and was close to the necrotic intima layer. In contrast, only in two out of six hemin-treated rats thrombus formation was observed, but these thrombi were only consisting of a few cells and independent from the intima layer. In the other cases of hemin-treated rats, no thrombi were found [181]. In parallel, the authors examined tin-protoporphyrin IX (SnPPIX)-treated rats, which revealed the same extent and characteristics of thrombus formation as the control rats. Since SnPPIX is a highly potent inhibitor of HO-1 and hemin a potent inducer of the same, the authors suggested that the ability of hemin to prevent or minimize thrombus formation might be due to induction of HO-1 expression [181], which subsequently exerts vasoprotective actions [182,183]. These results were later confirmed by another group [184]. Their data also suggested that an enhanced HO-1 induction prevents thrombus formation. 30 mice (septic C57BL/5 model) were treated with 50 µmol/kg hemin before sepsis and, consequently, thrombus formation was induced by the cecal ligation puncture procedure (CLP) [184]. The authors calculated the number of thrombi in the liver, lungs and kidneys, while using HE and MSB staining. In control mice, CLP greatly increased the number of thrombi, while in hemin-treated mice the number of thrombi was significantly reduced: ~50% (liver, HE), ~35% (liver, MSB), ~67% (kidney, HE), ~50% (kidney, MSB), ~50% (lung, HE & MSB) [184]. Additionally, they again observed markedly prolonged PT and aPTT of freshly drawn plasma of the treated mice. Zinc protoporphyrin (ZnPPIX) as a known HO-1 inhibitor counteracted the effect of heme [184]. Later, Hassaan et al. confirmed these results in CLP mice [185]. Therefore, these studies revealed HO-1 induction as a possible explanation for the anticoagulant role of heme, which was proposed by the observation of prolonged clotting times. Moreover, the authors even suggest the therapeutic use of heme in patients with venous thrombosis [180].

De Souza et al. (2017) determined (via rotational thromboelastometry) shortened clotting and clot formation times ex vivo after the addition of heme (30 µM) to whole blood, suggesting in contrast to most of the other studies a procoagulant role of heme [186]. Moreover, just recently, hemin applied at concentrations of 1–100 µM was shown to have no significant effect on the aPTT of pooled plasma from healthy volunteers. The addition of human serum albumin (0.1%) did not change these results [187].

Considering the reported changes of clotting times (in vivo and in vitro, Supplementary Table S2), the overall effect of pure hematin seems to be contradictory, describing

either an anticoagulant, no or a procoagulant effect of heme on these hemostatic parameters (Figure 2). Panhematin®, a mixture of hematin and sorbitol, consistently showed a tendency to an anticoagulant impact, whereas Normosang® (heme arginate) did not induce any changes of these hemostatic parameters (Supplementary Table S2; Figure 2).

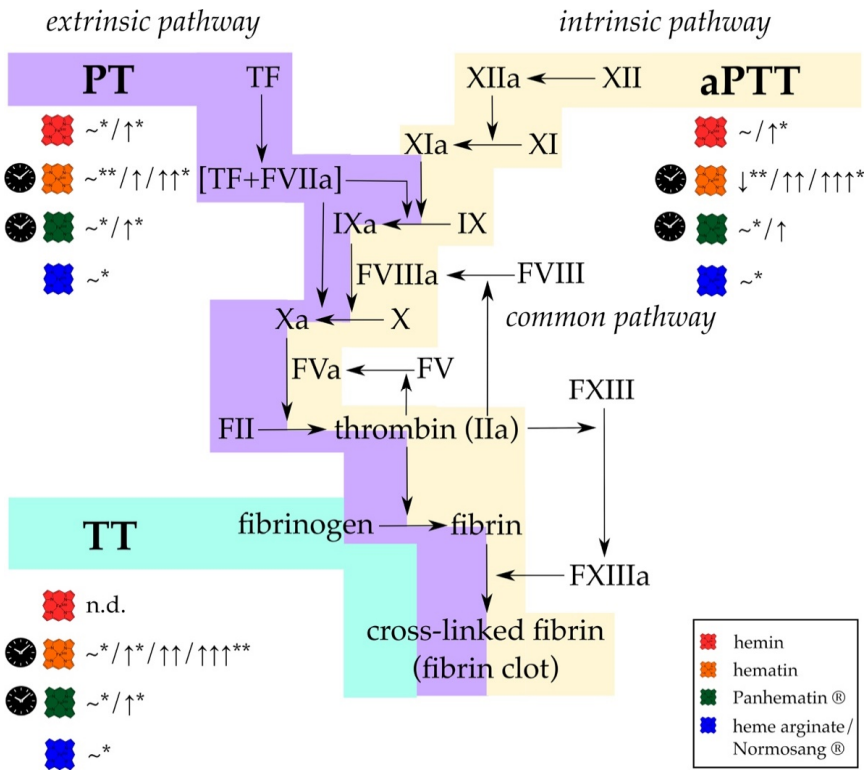

Figure 2. Different formulations of heme affect the results of standard coagulation diagnostic tests, (PT, aPTT, and TT) in vitro and in vivo. While PT is used to evaluate effects on the extrinsic and common pathway (violet), changes in the intrinsic and common pathways (yellow) can be determined using aPTT. With TT only the last step of fibrin generation and fibrin clot formation (turquoise) can be analyzed. Hemin (red symbol; concentration ranges: 50 mg/kg, 50 µmol/kg, 0.01–100 µM) either did not affect PT and aPTT or a slight prolongation was observed. TT was not determined (n.d.). In contrast, hematin (orange symbol; concentration ranges: 4–12 mg/kg, 3 nmol, 10–100 µg/mL) has been reported to induce significant prolongation of all clotting times. For Panhematin® (green symbol; concentration ranges: 4 mg/kg, 70–78 µg/mL), longer clotting times were also recorded, but to a lesser extent than with hematin. However, from detailed investigations of different researcher it can be assumed that just aged hematin and Panhematin® solutions can have such strong effects, whereas fresh hematin and Panhematin® solutions are ineffective (clock symbol) [156,157,175]. Heme arginate/Normosang® (blue symbol; concentration: 3 mg/kg) did not influence these diagnostic tests at all. FIIa = thrombin, FII = prothrombin, FV = factor V, FVa = activated factor V, FVIIa = activated factor VII, FVIII = factor VIII, FVIIIa = activated factor VIII, FIX = factor IX, FIXa = activated FIX, FX = factor X, FXa = activated FX, FXI = factor XI, FXIa = activated FXI, FXII = factor FXII, FXIIa = activated factor XII, FXIII = factor XIII, FXIIIa = activated factor XIII, n.d. = not determined, ~ = no effect, VWF = von Willebrand factor, ↓ = 30% decrease, ↑ = < 2-fold increase, ↑↑ > 2-fold increase, ↑↑↑ > 3-fold increase. * only observed in in vivo experiments; ** only observed in in vitro experiments.

In particular, the quality of the used hematin solutions should be considered. As reported, there might be a difference between the impact towards hemostatic parameters depending on whether a fresh or aged solution was infused [157]. In this context, fresh hematin solutions didn't show any prolongation of clotting and bleeding times. Quite evident, degradative products of hematin were shown to cause the observed anticoagulant effects [156,157,175]. In addition, the experiments were performed in different laboratories by different researchers and also reagents, solvents, concentrations and measuring devices (Supplementary Table S2). Discrepancies between the studies with hematin were suggested to be due to batch-to-batch variations or patients' diversity [178].

Altogether, it should be noted that between the first (1913) and the last (2020) report on the impact of heme (different formulations) on different clotting parameters there is a difference of more than 100 years (Supplementary Table S2). In total, presented studies of this section only involved 16 healthy test persons and 23 AIP patients. For that reason, there would be a need for more comparable studies in larger cohorts to unravel the actual effect of heme and its different formulations on clotting times as measured by standardized point-of-care test systems.

4. Heme Promotes Clotting Processes by Affecting Involved Cells

A variety of cell types, including platelets, endothelial cells, RBCs, and different kinds of leukocytes, is involved in the coagulation process [188]. Thus, it is not surprising that there are several studies which characterized the effect of heme on these cell types, linking heme and coagulation (Supplementary Table S3; Figure 3).

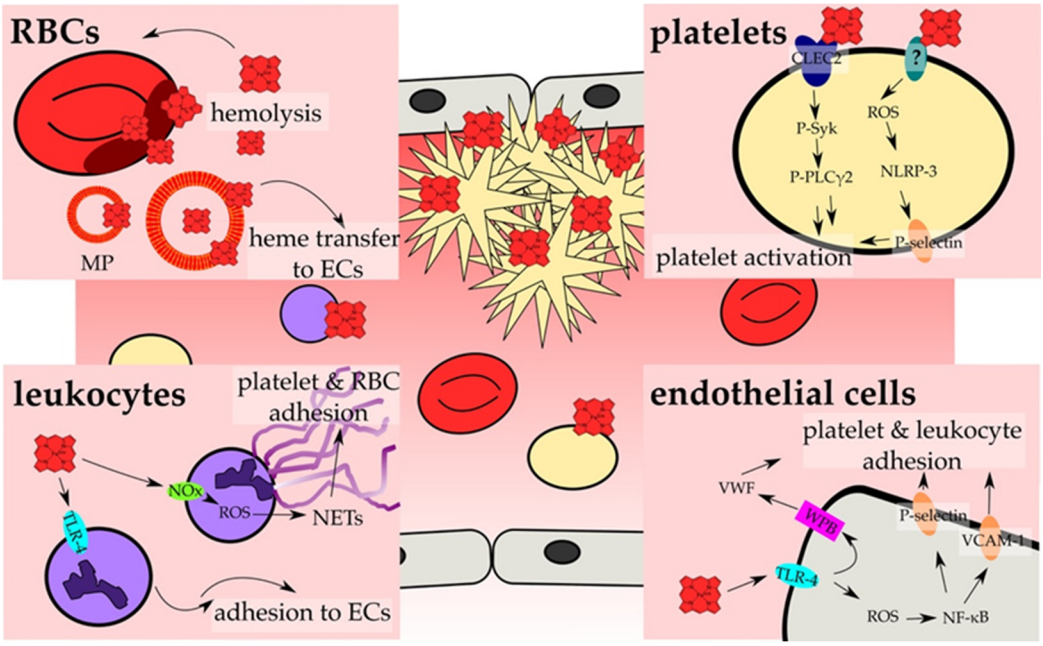

Figure 3. Heme activates cellular components of hemostasis. The main investigated pathways and consequences that result in the activation of cellular components of hemostasis and, thus, prothrombotic reactions by heme are depicted. On cellular level, hemostasis results from an interplay of RBCs (red, Section 4.3), platelets (yellow; Section 4.1), leukocytes (violet; Section 4.4) and endothelial cells (ECs, grey; Section 4.2). RBCs contribute to heme-induced hemostasis through the release of heme upon hemolysis, which can be further strengthened by heme itself (Section 4.3). Moreover, erythrocyte membrane particles (MP) incorporate and accumulate heme within the membrane, and allow for the transfer of heme to ECs (Section 4.2). These, in turn, become activated by heme in a TLR-4 -dependent manner (turquoise), which can lead either to the secretion of the contents of Weibel Palade bodies (WPBs; pink) (Section 4.2), among them VWF, or to ROS generation that triggers the increase of surface expression of adhesion proteins, such as P-selectin and VCAM-1 (orange) (Section 4.2). The exposure of those adhesion molecules as well as the secretion of VWF leads to the adhesion of platelets and leukocytes onto the endothelium. In contrast, activation of TLR-4 in leukocytes promotes the rolling and adhesion to ECs (Section 4.4). In addition, heme-induced NADPH oxidase (NOx; green)-dependent ROS generation in neutrophils can lead to NET formation, forming the scaffold for the adhesion of platelets and RBCs (Section 4.4). Finally, heme can also directly activate platelets. Two main mechanisms have been proposed. On the one hand, heme binding to CLEC2 was shown, leading to the phosphorylation of Syk (P-Syk) and PLCγ2 (P-PLCγ2) and eventually to the activation of platelets (Section 4.1). On the other hand, a ROS-dependent activation of the inflammasome via NLRP-3 has been demonstrated, which culminates in the expression of for example P-selectin, which again allows for the adhesion and activation of platelets (Section 4.1). Furthermore, the induction of ferroptosis (platelets) as well as apoptosis and necroptosis (endothelial cells) by heme has been demonstrated, which further might support the activation of endothelial cells and platelets (not shown; Sections 4.1 and 4.2).

4.1. Heme and Platelets

Platelets are essential mediators of blood coagulation. Upon tissue injury, vasoconstriction leads to exposure of collagen, which can direct platelet adhesion, activation, and aggregation. A platelet plug is formed, and the wound is closed [189]. An increased number or a functional defect of these cells can be associated with an increased risk of thrombosis [4,53].

Alongside a broad study regarding the impact of hematin on different cell counts in rabbits, Brown microscopically determined the platelet count upon hematin injection [142]. Even small doses (no precise data given) of hematin instantly induced a loss of platelets that remained for several hours. Accordingly, with higher doses (no precise data given) the effect was greater (reduction up to >50%). For example, a single dose of 25 mg/kg hematin reduced the platelet count by approximately 70% 1 h after injection. When the treatment was stopped, the platelet count returned to normal state. The author assumed that the impact on the platelet count is due to the destruction of platelets by hematin, and might be an explanation for the observed hemorrhage (Section 2) [142]. About 70 years later, the same was observed in AIP patients that were treated with hematin [153,169]. 12 h after administration the platelet count was decreased by approximately 57% (from 227,000/mm^3 to 98,000/mm^3) [153]. In another AIP case report, the platelet count was decreased by ~12% (from 176,000/µL to 154,000/µL), 7 h after the fourth injection of hematin (4 mg/kg) [169]. These observations were further confirmed by in vivo studies (Supplementary Table S3) [169]. In a study by Glueck et al., blood samples were collected 10 min after infusion of 4 mg/kg hematin. The highest plasma heme level was then detected (4 mg/100 mL), and the platelet count was already decreased by approximately 41% [169]. Subsequent in vitro studies on the platelet aggregation of platelet-rich plasma revealed that hematin (0.1 mg/mL) triggers platelet aggregation. Neither preincubation of hematin with 1 mM adenosine nor 1 mg/mL apyrase could prevent from aggregation. When adenosine (70 µM), apyrase (0.07 mg/mL) or adenosine triphosphate (ATP; 2.5 mM) were added to the plasma sample prior to hematin treatment, hematin-induced platelet aggregation was inhibited [169]. When platelets were washed or gel-filtered they still aggregated upon hematin addition (1 µg/mL). Acetylsalicylic acid (0.12 mg/mL) counteracted this effect of hematin (2 µg/mL), but with a higher hematin concentration applied (5 µg/mL) platelet aggregation was again induced. Moreover, Glueck and coworkers observed a dose-dependent induction of ^{14}C-serotonin secretion from platelets. For example, 0.1 mg/mL hematin induced a serotonin release of 10–12%, while 0.16 mg/mL hematin promoted a secretion of 82% [169]. ATP release by platelets was also increased by hematin. In conclusion, the authors suggested an hematin-induced platelet activation through the release of ADP [169]. Thus, the observed thrombocytopenia in patients correlates with the increased aggregation of platelets and not with a destructive effect of hematin (Supplementary Table S3). Additional treatment with anticoagulant agent heparin even worsened the coagulopathy in patients treated with hematin. Hence, the authors concluded that hematin has an anticoagulant effect [169].

In 1982, Peterson and coworkers suggested that epinephrine as a platelet agonist requires heme reduction in addition to binding the platelet α-adrenergic receptor to activate platelets [190]. Platelet aggregation was analyzed by using a dual-channel aggregometer, heme reduction by absorption determination at 558 nm (ε = 30 mM^{-1}) [190]. Typical reducing agents (e.g., ascorbic acid) were shown to reduce heme, but did not affect platelet aggregation due to their incongruous structure for binding to the receptor. Only epinephrine, which possesses both properties, was able to induce platelet aggregation in vitro. The authors refer to heme that occurs in form of a heme-protein-complex in the membrane of platelets, near to the receptor. To further support their hypothesis, platelet aggregation induced by epinephrine was analyzed in the presence of heme-binding compounds (iron chelators, i.e., phenanthroline and dipyridyl agents). Indeed, these compounds inhibited the epinephrine-mediated platelet aggregation [190]. Later, Malik et al. (1983) confirmed these results, while demonstrating the ability of exogenously added heme to enhance ADP-

and epinephrine-dependent platelet aggregation. In addition, binding of hemin to the platelet and the granule membrane was observed by ultrastructural localization via reaction of benzidine [191]. However, the underlying mechanism of heme-promoted platelet activation remained unresolved. Neely et al. (1984) aimed to decipher this mechanism with different approaches [192]. Again, hematin (2–5 µg/mL) was shown to promote aggregation of washed platelets. In comparison to the approach of Glueck et al. (1983), where hematin was added to platelet-rich plasma, a much lower (20- to 50-fold) hematin concentration was necessary to induce the same effect due to the absence of other plasma proteins that might scavenge hematin [169,192]. Microscopic ultrastructural analysis of the platelets confirmed the observed aggregation. The morphology of hematin-treated aggregated platelets was exactly the same as of platelets that were treated with typical platelet aggregation inducers (e.g., thrombin, collagen, ADP, and arachidonate). Moreover, hematin (5–10 µg/mL) triggered the production of thromboxane A_2 (92.7–187.3 ng per billion platelets) in a dose-dependent manner, which was associated with the observed platelet aggregation. The incubation of platelets with acetylsalicylic acid prior to hematin treatment completely impeded thromboxane A_2 generation [192]. The aggregation was independent of thromboxane A_2 production, because with the addition of acetylsalicylic acid in the experimental setup hematin still induced platelet aggregation compared to the situation without acetylsalicylic acid. In contrast, hematin-induced platelet aggregation was inhibited by verapamil, apyrase, prostacyclin, and prostaglandin to different degrees. As all of these compounds abolish platelet aggregation through a downstream upregulation of cyclic AMP levels, Neely et al. suggested that cAMP levels might serve as a key player in hematin-induced platelet aggregation [192]. In addition, the effect of verapamil was counteracted by an increase of the calcium concentration, so that extra-platelet calcium might be of importance in hematin-induced platelet aggregation as well. The authors discussed that hematin can support the influx of calcium ions and other divalent cations into platelets. In a separate approach, alongside $CaCl_2$, $MgCl_2$, $CoCl_2$, and $SrCl_2$ increased hematin-mediated platelet aggregation, whereas mono- and trivalent cations did not show any effect. Other compounds, in particular thrombin and collagen, strengthened the aggregation effect of hematin in a synergistic manner. In two thirds of the donors, heparin potentiated hematin-induced aggregation, but only when induced with low hematin concentrations (around 1 µg/mL; precise range not given). Therefore, the authors recommended not to use heparin in parallel to hematin therapy [192].

Later, it was shown that in patients with myelodysplastic syndrome an improvement of cytopenia, such as an increase of the total platelet count, was determined upon treatment with heme arginate (Normosang®; 2–3 mg/kg) [162,193]. The same was observed in AIP patients that received heme arginate (3 mg/kg) and hematin (Panhematin®) infusion (4 mg/kg) [165,178]. As opposed to the aforementioned studies, Green and Ts'ao also found a lower platelet aggregation in this context [178]. Furthermore, the ATP and ADP content of the platelets was decreased, which allows for the conclusion of a potential degranulation of the platelets, a fact that was not recognized during electron microscopic analysis [178]. In contrast, in 2000 a decreased platelet count (from 129000/mm^3 to 72000/mm^3) was determined in an AIP patient after hematin (Panhematin®; 4 mg/kg) administration [168]. At about the same time, an albumin (50 g/L)-heme (3 mM) mixture was demonstrated to have no effect on the platelet count of a blood suspension from rats [179] as well as on the ADP-stimulated activation of platelets as demonstrated by the proportion of PAC-1 positive platelets in human whole blood [194].

In 2004, Peng et al. shed light on the role of elevated HO-1 expression in the prevention of platelet-dependent arterial thrombosis [195]. The authors observed that heme (15 mg/kg; intraperitoneal injection; twice daily) significantly accelerated platelet-rich thrombi formation in HO-1 knockout mice after stimulation with $FeCl_3$, suggesting that hemin itself might have a prothrombotic effect. Moreover, the authors suggested a correlation of the heme-induced increase of oxidative stress and the observed accelerated thrombosis in HO-1 knockout mice, which cannot be prevented in the absence of HO-1. Upon hemin

administration, platelet cGMP levels were significantly increased in wild-type and HO-1 knockout mice. However, when only hemin (40 mg/mL) was administered without pre-treatment with $FeCl_3$, no thrombotic phenomena were observed, neither in wild-type nor in HO-1 knockout mice. Therefore, the authors concluded that hemin itself might not affect the formation of platelet-dependent thrombi in their experimental setup [195].

After intraperitoneal injection of 50 mg/kg hemin in rats, Desbuards and colleagues determined a significant increase of the platelet count, but there was no sign of thrombus formation [181]. A dual role of hemin and hematin was suggested, exerting both anti-coagulant and procoagulant functions. However, the potential relevance thereof is not yet understood. Heme might first promote platelet activation and second stimulate CO release through its degradation by HO-1, which further leads to the inhibition of platelet aggregation, e.g., through stimulation of soluble guanylate cyclase and upregulation of cGMP [181].

Since 2018, a ROS-dependent activation of platelets by heme was considered (Supplementary Table S3; Figure 3) [196]. In the course of their studies, Naveen Kumar et al. (2018) analyzed the cytotoxic effect of hemin on platelets, revealing a reduction of cell viability along with an elevated lactate dehydrogenase (LDH) release upon hemin treatment. Furthermore, platelet morphology was strongly affected. While lower concentrations (5–10 µM) provoked filopodia-like structures, higher concentrations (25 µM) led to the damage of the platelet membrane. Neither cytochrome c release nor caspase-3 activation was induced by heme, confirming a non-apoptotic cytotoxic effect [196]. Necroptosis was also disproved, since the necroptosis-specific inhibitor necrostatin-1 was not able to counteract heme-mediated death of platelets. However, when human platelets were treated with hemin (25 µM), an approximately six-fold increase of cytosolic ROS, depleted glutathione levels, and massive lipid peroxidation were detected. These effects were associated with an increased expression of HO-1 and subsequent elevated platelet iron levels. Thus, ferroptosis was suggested as a cause for heme-induced platelet cell death [196]. Furthermore, heme treatment of platelets was followed by elevated P-selectin levels, which is a marker for platelet activation, and the formation of platelet microparticles (PMPs). Interestingly, PMP generation was observed in several prothrombotic diseases, among these SCD [197]. The potent ferroptosis inhibitor ferrostatin-1 prevented all observed heme-mediated effects, observed in this study, suggesting that platelet activation and cell death are induced via ferroptotic pathways upon heme treatment [196]. In 2019, Naveen Kumar et al. analyzed these heme-mediated effects also in mice [198]. Thereby, a significant reduction of the total platelet number was observed. Moreover, for the first time it was shown that heme activated platelets through inflammasome activation in a NLRP-3 manner, driven by heme-induced ROS generation [198]. Interestingly, both heme-mediated ferroptosis and activation of platelets were prevented by melatonin, suggesting antioxidant melatonin as a potential drug for the treatment of thrombosis under hemolytic conditions [198].

Recently, Bourne et al. (2020) suggested that in contrast to endothelial cells (Section 4.2) platelets are activated by heme in a Toll-like receptor 4 (TLR-4) independent manner [199]. Instead, this process seems to be directed by an immunoreceptor-tyrosine-based activation motif receptor (ITAM receptor) based signaling pathway. Hemin at low concentrations (<25 µM) stimulates phosphorylation of the protein tyrosine kinase Syk and phospholipase C gamma 2 (PLCγ2). Indeed, addition of recombinant CLEC2 blocked platelet activation. Therefore, the authors concluded a crucial role of this receptor in heme-triggered platelet activation (Figure 3). Moreover, direct binding of heme to recombinant CLEC2 was demonstrated by applying spectroscopic methods, revealing a heme-binding affinity of ~200 nM. Heme-triggered aggregation of platelets was independent from oxidative stress, as the antioxidant N-acetyl cysteine could not prevent it. Interestingly, at higher hemin concentrations (>25 µM) platelet aggregation seemed to be independent from Syk. For that reason, the authors suggested that these high hemin levels might result in agglutination. Through the potency of recombinant CLEC2 in prevention of heme-driven platelet activation, it is suggested as a potential therapeutic agent against thrombosis in hemolytic patients [199].

4.2. Heme and Endothelial Cells

As the primary source of different molecules that participate in the clotting process, endothelial cells are pivotal for the regulation of blood coagulation. In healthy states, endothelial cells possess an anticoagulant and, in turn, antithrombotic nature through the secretion of various anticoagulants, such as thrombomodulin, EPCR or platelet inhibitors (e.g., NO, prostacyclin) [200]. Upon vessel injury, endothelial cells undergo activation which is followed by the expression of procoagulant proteins, such as TF and VWF. Subsequently, these proteins can initiate the coagulation cascade and trigger platelet activation, respectively [200]. Accordingly, in case of functional abnormalities the properties of endothelial cells may tend towards a prothrombotic nature [200].

In 1984, Neely et al. detected dose-dependent morphologic changes of bovine aortic endothelial cells (BAECs) after incubation with hematin (2–40 µg/mL, diluted in HEPES buffer) [201]. These alterations were reversible and marked by bulging, surface vesiculation, and cell retraction as determined with inverted phase-contrast light and scanning electron microscopy. When hematin was diluted in plasma, 40 µg/mL hematin caused the same extent of morphologic changes as 10 µg/mL hematin diluted in HEPES buffer. Thus, Neely and coworkers suggested that only unbound heme can affect endothelial cells [201]. In addition, the hematin-induced morphologic changes were attributed to hematin-triggered stimulation of contractile elements of the cells. These changes were also reversible, since the endothelial cells returned to their usual appearance after removal of hematin. While BAECs exposed to up to 40 µg/mL hematin did not show any sign of increased detachment, incubation with 100 µg/mL hematin resulted in a significant rise of cell detachment (from ~7.9% to ~13.0%). However, these effects of hematin might lead to an exposure of subendothelial structures with thrombotic consequences (Supplementary Table S3). This was further supported by an increased platelet adhesion to hematin-exposed BAECs [201]. Untreated BAEC monolayers bound less than 5% of platelets from a suspension of washed human platelets. When incubated with hematin (40 µg/mL) the platelet adhesion to endothelial cells was two times higher. Furthermore, addition of hematin to the platelet suspension increased adhesion even more. Via scanning electron microscopic examination, sites of adhesion were identified as matrix materials, plastic and the by hematin superficial roughened endothelial cells [201]. As a side effect, aggregates in the platelet suspension were observed after hematin addition, confirming the previously described results on the impact of hematin on platelets (Section 4.1). Finally, Neely et al. considered their observations as a potential basis for the clinically noticed thrombophlebitis and thrombocytopenia (Supplementary Table S3) [201].

Balla and coworkers observed alterations of porcine aortic endothelial cells (PAECs) upon exposure to hemin [202]. After fast heme uptake and accumulation within the plasma membranes, the cells were more sensitive towards oxidative stress as induced for example by H_2O_2, implicating a possible role in atherogenesis or hemorrhagic injury. This effect is mainly driven by heme-mediated LDL oxidation and was effectively prevented by the heme scavenger hemopexin [202,203].

In a study that aimed for the investigation of causes for iron-induced endothelial injuries as a consequence of hemolysis, Woollard and colleagues (2009) analyzed the effect of hemin for comparison [204]. Thereby, an isolated mouse aorta was perfused with 1 mM hemin resulting in a mild denudation of the endothelium, which was accompanied by collagen exposure and platelet aggregation [204]. A more extensive reaction of endothelial cells towards hemin exposure (10–50 µM) was demonstrated in 2012 [205]. Characterized by a concentration-dependent increase of LDH release, hemin induces apoptosis of BAECs. Only the administration of hemin with the highest applied concentration (50 µM) resulted in an increase of ATP. Moreover, apoptosis was accompanied by a significant appearance of cleaved caspase 3 and caspase 9 and, thus, is mediated via the mitochondrial intrinsic cell death pathway. Therefore, heme-induced mitochondrial damage was expected to occur prior to the initiation of endothelial cell death [205]. A heme-mediated decline of mitochondrial membrane potential and the suppression of basal respiration in BAECs

further supported this assumption. Moreover, it was shown that heme-induced lipid peroxidation plays a major role in heme-mediated cell death. Here, hemin exposure of BAECs resulted in the formation of carbonyl adducts as well as thiol oxidation of proteins [205]. Interestingly, the level of LC3-II, the membrane-bound form of the central protein of autophagy LC3, is increased in BAECs 2 to 4 h after treatment with 25 µM hemin. In some cells also mitophagy was observed. Generated autophagosomes include lipid-protein adducts, suggesting a protective mechanism against hemin toxicity [205].

In 2014, Vercellotti and coworkers [206] infused heme (prepared as Panhematin®; 0.4–32 µmol/kg) into transgenic sickle mice (i.e., NY1DD) and wild-type mice leading to the development of vaso-occlusion in subcutaneous venules (19.7–38.9% stasis) only in sickle mice [206]. Pre-existing chronic hemolysis in sickle mice in comparison to wild-type mice might be the cause for the aforementioned observations, since heme-mediated vaso-occlusion clearly correlates with plasma heme levels. In fact, heme-induced stasis is associated with a high degree of adhesion molecules' expression, such as of P-selectin and VWF. Heme-mediated initiation and potentiation of the expression of adhesion proteins (Section 3) was observed in vivo on vessel walls for both, sickle and wild-type mice. Moreover, in vitro heme exposure (20 µM) of HUVECs lead to the activation of the same with an increased expression of adhesion molecules on the cells' surface, which might be dependent on NADPH oxidase and its activator protein kinase C (PKC) [206]. The authors identified TLR-4 signaling as the major trigger for vaso-occlusion in sickle cell mice, leading to NF-κB activation and, subsequently, expression of various adhesion molecules and degranulation of Weibel-Palade bodies [206] (Figure 3). However, as previously demonstrated for the heme-driven LDL oxidation in endothelial cells [202,203], also heme-mediated stasis was prevented by equimolar co-administration of hemopexin [206]. Furthermore, protoporphyrin (PPIX) antagonized heme-induced stasis, when intraperitoneally administered 1 h prior to heme.

Camus et al. (2015) realized that under the hemolytic conditions of SCD heme itself is transferred to the endothelium by circulating erythrocyte membrane microparticles (MPs), thereby promoting vaso-occlusion [207]. For the first time, it was demonstrated that a significant portion of cell-free heme (~one-third) binds to cell membrane fragments (Supplementary Table S3). In vitro, incubation of HUVECs with SCD-derived heme-laden MPs and synthetic heme-laden multilayer vesicles (MLVs) led to the incorporation of heme into HUVECs. In comparison to control erythrocyte MPs (contain ~20 nM heme), SCD erythrocyte MPs (contain ~65 nM heme) transferred heme in a 4-fold more efficient manner. Moreover, both SCD erythrocyte MPs and synthetic heme-laden MLVs triggered production of ROS and induced apoptosis, leading to strong endothelial toxicity and, thus, endothelial injury [207]. Since TLR-4 blocking resulted in a nearly complete inhibition of heme-laden MP-triggered ROS production, the authors concluded that the observed effects might be mediated in a TLR-4-dependent fashion, as already suggested by others for heme-mediated effects on endothelial cells [206] (Figure 3). In addition, hemopexin (2 µM) and the phosphatidylserine antagonist annexin-a5 (10 µg/mL) restrained heme transfer to HUVECs, ROS production and apoptosis. Hemopexin's preventive impact was explained by its heme-scavenging properties. Furthermore, it seems to be capable of removing heme from MPs to a certain extent. The blockage by the phosphatidylserine antagonist prompted the authors to investigate a potential relevance of phosphatidylserine. Subsequent in silico studies suggested that MP-exposed phosphatidylserine binds heme with the help of two calcium ions. Further support of this prediction was gained by the fact that removal of calcium ions by complexation resulted in a reduced heme association to MPs [207]. However, in SAD mice (S-Antilles-D Punjab Hb-expressing SCD mouse model) heme-laden MPs induced kidney vaso-occlusions, while perfusion of 100 nM heme or MPs (300 MPs/µL) isolated from SAD mice was followed by a massive reduction of endothelial acetylcholine-dependent vasodilation in mouse mesenteric resistance arterioles, which was counteracted when MPs were preincubated with 1 µM hemopexin.

While Camus et al. (2015) observed heme-triggered apoptosis in HUVECs [207], in the setting of Singla et al. (2017) necroptotic pathways instead of apoptotic pathways were induced by heme [208]. Human lung microvascular endothelial cells (HLMVECs) showed a TLR-4-dependent loss of endothelial barrier stability upon hemin treatment (5–100 μM) as shown by a decrease of trans-endothelial electrical resistance along with an increase of monolayer permeability. These effects were abrogated by addition of a TLR-4-inhibitor, as well by the antioxidant N-acetylcystein and the iron chelator deferoxamine. Therefore, not only the activation of TLR-4-dependent pathways, but also heme-mediated ROS production might be important for these observations. In addition, heme's iron moiety seems to be essential [208]. However, programmed cell death was triggered by hemin (40 μM) in a TLR-4- and ROS-dependent but caspase-3 independent manner. Necroptosis was confirmed by an increased activation of the mixed lineage kinase domain-like (MLKL) as a consequence of TLR-4 activation and ROS production [208] (Figure 3). The authors suggested that these molecular processes might contribute to the vaso-occlusive crises of SCD patients [208].

In context of atypical hemolytic uremic syndrome (aHUS), May et al. (2018) found different explanations for the procoagulant state of patients as well as for the complement activation on the level of the endothelium in vitro and in vivo (Supplementary Table S3) [209]. When macrovascular cells (i.e., HUVECs) were exposed to hemin (12.5–50 μM), increased HO-1 gene and protein expression as a mechanism of heme detoxification was observed. Moreover, the expression of the transmembrane receptor thrombomodulin was significantly upregulated. Due to its role in the anticoagulant protein C pathway, these changes might not only be relevant for heme-mediated regulation of the complement but also coagulation system [209]. However, these characteristics occurred to a lesser extent in microvascular endothelial cells (i.e., HMECs, GENCs, HRGECs). Thus, in particular the microvascular glomerular cell types (i.e., GENCs, HRGECs) were more susceptible towards heme-triggered complement activation, characterized by increased deposition of complement component 3 (C3). This is in good agreement with the commonly found microvascular thrombotic microangiopathic lesions in aHUS [209]. Overactivation of the complement system was also observed in mice, in particular in the kidney glomeruli, as a consequence of intraperitoneal hemin injection (40 μmol/kg). While thrombomodulin levels were elevated in skin and large liver vessels, the lung microvasculature showed decreased levels of thrombomodulin. Therefore, the authors suggested a procoagulant and complement-activating role of the microvascular endothelium under hemolytic conditions, which might be prevented by compounds with blocking properties towards heme toxicity [209]. Indeed, heme-induced (50 μM) elevated P-selectin expression on the surface of HUVECs as well as complement activation were prevented by hemopexin (5 μM), but not by HSA [210]. As Camus et al. (2015) already described [207], here again the relevance of RBC-derived MPs as heme carriers was demonstrated. Subsequent heme-dependent physiological consequences were again completely inhibited by hemopexin [210]. Heme-induced activation of endothelial cells was shown to not only be accompanied by an increased expression of adhesion proteins, like P-selectin and ICAM-1, but also by an elevated adhesion of HbS-RBCs to the endothelial cells, whose relevance was also demonstrated in SCD patients with vaso-occlusive crises [74].

4.3. Heme and RBCs

RBCs can participate in the blood coagulation process through provision of phosphatidylserine and microparticles, and subsequent initiation of thrombin generation [211]. Moreover, RBCs are incorporated into the thrombus through interaction with platelets and endothelial cells, which prevents from clot resolution. Therefore, quantitative and qualitative changes of RBCs, such as under hemolytic conditions, are accompanied by a higher incidence of thrombosis [32,211].

Microscopic studies of Brown (1913) revealed that the RBC count was affected by hematin administration to a much lesser extent in comparison to the platelet or leukocyte

count [142]. However, while doses below 15 mg/kg hematin caused a decrease of the RBC count in rabbits with fast regeneration in only few cases, a dose of 20 mg/kg intravenously injected hematin directly induced a decline of the RBC count. Furthermore, Brown noticed that a daily injection of 10 mg/kg hematin was followed by a decrease of the RBC count by approximately 60% [142]. In addition, the RBCs were characterized by irregularities in size, variability of color, and the presence of immature cells, in particular basophilic cells. The reduction of the RBC count was assigned to hemolysis that was directly initiated by hematin itself. This assumption was supported by the observed hemoglobinemia [142]. In later published case reports of AIP patients, the injection of hematin was shown to induce a slight decline of the hematocrit without any obvious morphological changes of RBCs (Supplementary Table S3) [153,169]. At about the same time, other reports supported the hemolytic impact of heme on RBCs as kind of a feedback loop that might explain the decreased RBC count in the presence of high labile heme levels [212,213]. Heme ($\geq$500 nM heme; lower concentrations not tested) accumulates in the RBC membrane, thereby inducing loss of intracellular potassium, which leads to hypotonic lysis of RBCs. Subsequently, swelling of RBCs was observed [212]. This was further accompanied by a decrease of glutathione, ATP, as well as hemoglobin levels. It was found that 5 µM heme was capable of inducing 50% hemolysis of RBCs after 2.5 h of incubation [212]. In another study, heme-induced RBC membrane instability and subsequent lysis was attributed to heme (20–500 µM)-triggered conformational alterations of cytoskeletal protein spectrin and protein 4.1 [214]. More recently, this was further supported by spectroscopic binding studies that aimed to characterize the heme-binding capacity of dimeric spectrin, revealing a K_D value of 0.57 µM [215]. Molecular docking suggested a specific binding site within the SH3 domain of erythroid spectrin [215].

Interestingly, the concentration of heme within the membrane of aged RBCs was demonstrated to be increased. Shaklai et al. (1985) proposed a potential role of heme in hemolysis and, thus, removal of aged RBCs from circulation [213]. Albumin was able to extract heme from RBCs membranes [213]. Moreover, heme-induced hemolysis (e.g., 50% hemolysis in case of 40 µM heme applied) was prevented in the presence of 1% of albumin, as a classical heme-scavenging protein.

In 2003, a mixture of albumin (50 g/L) and heme (3 mM) was shown to not induce any change of the RBC count, which again demonstrated the protective role of albumin towards heme toxicity [179]. Noticeably, the same was observed when hemin (50 mg/kg) was injected daily in normal Wistar rats—neither hematocrit nor the RBC count was significantly changed (Supplementary Table S3) [181].

4.4. Heme and Leukocytes

Leukocytes play a role in coagulation through the production of cytokines that can modulate the expression of pro- and anticoagulant proteins as well as adhesion molecules [216,217]. In addition, they can directly interact with other vascular cells, including platelets and endothelial cells. Dysregulation of leukocyte activation or abundance may thus lead to thrombotic complications [217]. In particular, monocytes and neutrophils contribute to a procoagulant state, in particular through an elevated expression of the procoagulant TF on their surfaces (Section 5.8) [218].

In 1911, W. H. Brown reported a large number of polymorphonuclear leukocytes after hematin and hemin injection in rabbits, as determined at sites of accumulation of the pigments by microscopic analysis [138]. A few years later, the author extended these experiments and directly compared the effect of hematin intoxication with those of the control injections [142]. Leukocytosis (e.g., ~1.4-fold total leukocyte count) was, thus, observed in rabbits characterized by an increase of large (e.g., ~5.6-fold) and small (e.g., ~1.5-fold) mononuclear leucocytes. In addition, there was a decrease of eosinophiles and an increase of basophiles. Cells were counted 11 days after a single injection of 10 mg/kg hematin. Four days after a daily dose of 20 mg/kg hematin, a marked increase of large mononuclear leukocytes (~10-fold) and a higher number of polymorphonuclear cells (~7.8-

fold) was detected. The total number of leukocytes was 4.6-fold increased. Not exclusively, but partially these effects were attributed to the alkaline solution [142]. Similarly, in patients with myelodysplastic syndromes a great rise of neutrophils after treatment with heme arginate (Normosang®; 2–3 mg/kg) was observed [162,193]. However, a direct connection of heme-neutrophil interaction with coagulation was proposed by Smith and Winslow (1992), when they identified heme as a stimulating agent of the procoagulant activity of isolated human peripheral blood mononuclear cells (PBMCs) [219]. Furthermore, they suggested that the procoagulant effect of stroma-free hemoglobin solutions might be also due to heme itself [219].

Considerably later, it was shown that ^{111}In-labeled leukocytes quickly migrated and accumulated in different organs of mice, such as liver and spleen, after intravenous infusion of hemin (intravascular concentration: 750 µM) [220]. In parallel, lesions were registered supporting the role of heme as an inflammatory mediator. Interestingly, Arruda and coworkers demonstrated a significant delay of neutrophil apoptosis by heme (1–50 µM) in vitro, which is inevitably associated with the de novo synthesis of antiapoptotic proteins (e.g., Bcl-x_L, interleukin (IL)-8) [221,222]. A particular role of the Ras/Raf/MAPK and phosphoinositide 3 kinase (PI3K) pathways for the heme-mediated protection of neutrophils was suggested, since inhibitors of these pathways completely reversed the heme effect. Heme itself promoted extracellular signal-regulated kinase (ERK)-2 translocation to the neutrophil nucleus and triggered PKC-dependent protein kinase B (Akt) phosphorylation, a key step in PI3K/Akt signaling, and, thus, the degradation of proapoptotic proteins (e.g., Bad) [221,222]. In the presence of SnPPIX, an inhibitor of heme oxygenase, the protective effects of heme were partially revoked. The authors suggested an important role of heme degradation products in the mediation of neutrophil survival. However, neither biliverdin nor bilirubin was able to protect neutrophils from apoptosis [221]. In addition, ROS production and consequently redox potential changes of the cells might be crucial for the heme-mediated antiapoptotic effect on neutrophils, very likely through NF-κB activation by heme [221,222]. In the presence of albumin, neither a difference of the chemotactic effects of heme on neutrophils [222] nor a change of the total leukocyte number in blood suspensions from rats (Supplementary Table S3) [179] were observed, displaying the protective role of albumin towards heme's effects on leukocytes.

At the same time, Wagener et al. (2003) confirmed the accumulation of large amounts of heme (no precise data available) at sites of injuries in a wound-healing model in rats [223]. As a consequence, an enormous infiltration of leukocytes occurred, mainly consisting of granulocytes (after 1 day) and macrophages (after 3 days) [223]. The number of lymphocytes did not change. These observations were confirmed when 750 µM hemin was intradermally injected in rat mucosa [223]. There is evidence that the leukocyte influx is triggered by inflammatory, chemokine-dependent pathways, as for example granulocytes exposed to heme exhibited increased IL-8 expression [222]. In order to unravel the role of heme oxygenase (HO) in heme-induced leukocyte recruitment, the mucosa was pre-exposed (24 h) to the HO inhibitor tin mesoporphyrin (20 µM) prior to hemin treatment [223]. An even more aggravated number of granulocytes and macrophages infiltrated into the mucosa, confirming again the protective role of HO [223]. Desbuards et al. (2007) also observed a higher number of leukocytes in rats, after they were treated either with 50 mg/kg hemin per day or with a mixture of hemin (50 mg/kg per day) and the HO-1 inhibitor SnPPIX (60 mg/kg per day) for a period of seven days [181]. While other effects of hemin were attributed to an induction of HO-1 and consequent prevention of thrombosis, these data are not in accordance with this hypothesis but fit well to the observations of Wagener et al. (2003) (Supplementary Table S3) [181,223].

A few years later, Belcher et al. (2014) noticed increased rolling and subsequent adhesion of leukocytes to the endothelium in subcutaneous venules of sickle mice (NY1DD) after infusion of heme (3.2 µmol/kg; prepared as Panhematin®) [206]. This occurred in a TLR-4-dependent manner, thus, supporting the hypothesis raised by Arruda et al. (2004) that already suggested a NF-κB-dependent signaling as the underlying mechanism

for heme-induced effects on leukocytes [206,221]. At the same time, Chen et al. (2014) identified heme as an inducer of neutrophil extracellular traps (NETs) generation in vitro and in vivo [224]. NETs were shown to be capable of capturing RBCs and platelets as well as to be part of venous thrombi [225,226], hence why these findings are of great importance to understand the role of heme as a procoagulant molecule. In SCD mice, the authors observed NET formation in pulmonary blood vessels, which were increased when pre-stimulated with the cytokine TNFα. The same was observed in TNFα-treated hemizygous mice after intraperitoneal hemin injection (50 µmol/kg). TNFα-primed wild-type bone marrow neutrophils were stimulated with 10–20 µM hemin and produced NETs in a dose-dependent manner (Figure 3). Neither PPIX nor ZnPPIX induced the formation of NETs in primed neutrophils. As already described elsewhere for the heme-mediated activation of neutrophils [130,222], NET formation occurred as a consequence of intracellular upstream of ROS by heme (Figure 3). Since neither heme nor TNFα alone induced NET formation, the authors suggested that a predisposal of neutrophils by proinflammatory cytokines is necessary to become sensitive for heme-mediated NET generation [224]. Hemopexin was able to abrogate the observed NET generation by scavenging heme, whereas addition of HSA only slightly reversed the effect [224]. In 2017, heme-induced NET formation was fluorometrically quantified demonstrating a dose-dependency in the presence of 1.5–15.3 µM hemin [227]. In human neutrophils, nuclear swelling and slight structural NET-like changes were observed upon hemin treatment (7.7–15.3 µM). The authors confirmed that heme-mediated NET production was ROS-dependent, more precisely NADPH oxidase-derived ROS-dependent. In contrast to the observations of Belcher et al. (2014) concerning neutrophil adhesion [206], NET formation was not TLR-4-dependent (Supplementary Table S3) [227].

5. Direct Influence of Heme on Coagulation Proteins

Early signs of heme as an actor in blood coagulation with partially controversial results and unresolved questions led to the assumption that there must be other relationships apart from the effects on hemostatic cellular components [142,153]. Hence, researchers started to consider a direct interference of heme with the clotting cascade [169]. In this context, heme-mediated regulation of protein expression levels or transient heme binding to proteins with functional consequences were suggested [97,169,178,228]. This is applicable to several procoagulant (e.g., TF and fibrinogen) and anticoagulant (e.g., activated protein C (APC)) proteins, as described in the following (Supplementary Table S4, Figure 4).

5.1. Heme Interaction with Fibrin(ogen)

In the course of the development of porphyrins as photosensitizing agents for photodynamic therapy, W. H. Howell observed in 1921 that hematoporphyrin IX prepared from hemin induced a change of fibrinogen's solubility [229]. As a consequence, thrombin was unable to convert fibrinogen in its coagulable form fibrin [229]. Later, this was confirmed by others, who suggested direct binding of hematoporphyrin to fibrinogen [230,231]. Musser et al. (1979) supported this assumption by chromatographic techniques [232]. In contrast, no binding to fibrin, neither in its crosslinked (with factor XIIIa) nor in its non-crosslinked form, was observed [233].

In 1983, Glueck et al. demonstrated that intravenous hematin (4 mg/kg) infusion in an AIP patient resulted in a decline of fibrinogen level (from ~2.8 mg/mL to ~1.9 mg/mL) and a rise of FDP (~2-fold), 10 min after infusion [169]. Complete recovery was reached 48 h after infusion. Moreover, the authors showed that fibrin polymerization was not affected by hematin upon preincubation of fibrin monomers with hematin (20–90 µg/mL) [169].

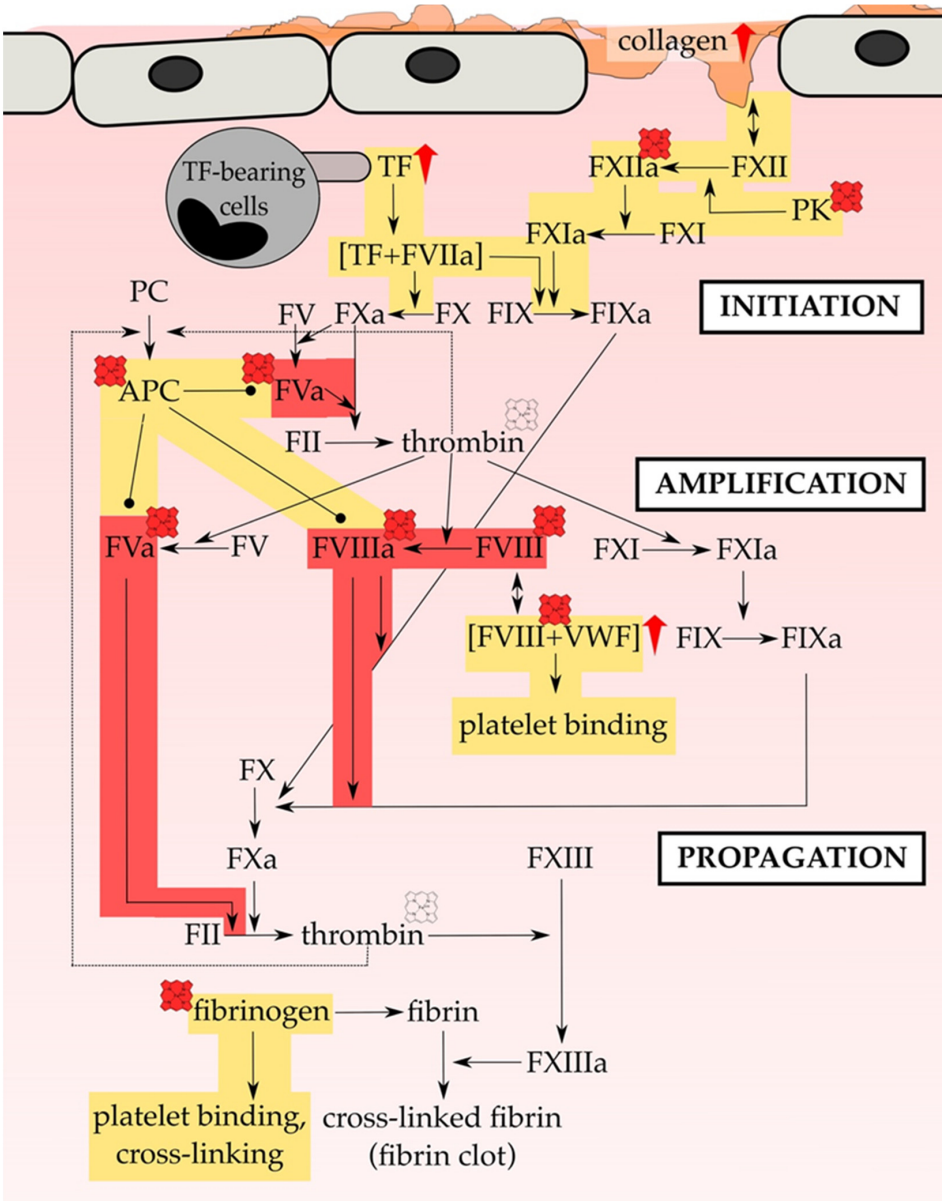

Figure 4. Heme promotes plasmatic hemostasis. Heme can directly affect various proteins of the blood coagulation cascade. While usually activated through the exposure of TF (e.g., by monocytes or the subendothelium), in pathologic thrombotic situations hemostasis activation through the exposure of negatively charged surfaces (e.g., of collagen) plays a supportive role. Indeed, initiation, amplification and propagation of hemostasis on the surface of cells (TF-bearing cells and platelets) is targeted by heme, either through upregulation of proteins' expression level (red arrow) or regulation of proteins' function (heme symbol). Direct heme-binding with functional consequences was only demonstrated for APC, FVIII(a) and fibrinogen. Contradictory results were obtained in case of the impact of heme on the activity of thrombin (pale heme symbol). The investigations of more than 35 years research allow for the assumption that heme is able to initiate hemostasis via both the upregulation of TF expression on leukocytes and endothelial cells as well as of collagen in the subendothelium. Most of the analyzed interactions tend to a procoagulant/prothrombotic (yellow) impact of heme. In contrast, heme-induced FVIIIa and FVa inactivation is exclusively described leading to anticoagulant (red) consequences. FVIII and FV are central cofactors of the coagulation cascade. Thus, the inactivation of FVIIIa and FVa by heme could constitute kind of a control center of heme-mediated initiation, amplification and propagation of the coagulation process. Plasma level changes of clotting factors that were recorded in humans upon heme infusion are not included. APC = activated protein C, FIIa = thrombin, FII = prothrombin, FV = factor V, FVa = activated factor V, FVIIa = activated factor VII, FVIII = factor VIII, FVIIIa = activated factor VIII, FIX = factor IX, FIXa = activated FIX, FX = factor X, FXa = activated FX, FXI = factor XI, FXIa = activated FXI, FXII = factor FXII, FXIIa = activated factor XII, FXIII = factor XIII, FXIIIa = activated factor XIII, PC = protein C, PK = plasma kallikrein, VWF = von Willebrand factor.

Based on the first results indicating a potential interaction of hematin with fibrinogen, Green and colleagues analyzed direct binding by size-exclusion chromatography (SEC) with a sephadex G-200 column [174]. Co-elution of fibrinogen and hematin was observed, which was interpretated as hematin binding to fibrinogen. Within this study, hematin was solved in 0.25% sodium carbonate solution for 24 h prior to usage. Due to other studies that followed shortly after, the formation of anticoagulant acting heme degradation products was assumed (Section 3) [156,157,175]. At about the same time, the same group demonstrated that upon a 30 min preincubation with hematin (stored at 4 °C in sodium carbonate buffer) fibrinogen binding to gel-filtered platelets was induced [192]. Again, hematin was not used in a fresh state, but, in contrast to the previous reports, here, hematin acted in a procoagulant manner.

In line with the unaltered clotting times in the presence of heme arginate (Section 3, Figure 2), 3 mg/kg heme arginate (Normosang®) did not cause any change of fibrinogen, FDP, and fibrinopeptide A plasma levels in healthy test persons [177].

More than 20 years later, Nielsen et al. (2011) suggested at least one permanently bound heme group in fibrinogen [234]. This hypothesis was only based on the finding of the $[M + H]^+$ ion of heme after LC-MS/MS analysis of fibrinogen and needs further evidence. Moreover, addition of nitric oxide/hydroquinone prevented from CORM-2-mediated procoagulant effects, thus suggesting heme-mediated carbon monoxide sensing by fibrinogen [234]. The presence of one or more permanent heme-binding sites in fibrinogen, however, has not yet been proven so far.

At the same time, Barrera et al. (2011) suggested fibrinogen as a hemozoin-binding protein [235]. Exposure of hemozoin (isolated from *Plasmodium falciparum* cultures) with plasma resulted in an enrichment of host fibrinogen in hemozoin. Binding of one molecule fibrinogen to approximately 25,000 hemozoin-heme molecules was estimated from SDS-PAGE [235]. Further experiments are required to confirm and characterize the proposed direct interaction and the ratio between the interaction partners. In human monocytes, the putative hemozoin-fibrinogen complex caused TLR-4-mediated oxidative stress. Furthermore, binding of different cell types, such as platelets and endothelial cells, via fibrinogen receptors might play a role in malaria pathogenesis [235].

In 2013, K. Orino immobilized fibrinogen on Sepharose 4B beads and demonstrated binding of hemin (10 μM) to bound fibrinogen spectroscopically [236]. Moreover, the author observed that fibrinogen-bound hemin still exerted peroxidase-like activity, which was comparable to unbound heme [236].

Direct heme binding of fibrinogen was further confirmed by Ke and Huang (2016), who demonstrated a shift of the Soret band of hematin (20 μM) to 410 nm upon complexation by fibrinogen [237]. Raman spectroscopy allowed for the assumption of a hexacoordinated complex. Functional studies under non-thermal plasma exposure revealed that hematin (50–500 μM)-treated blood and plasma showed rapid superficial clot layer formation. This layer consisted of cross-linked protein polymers with high molecular weight (>245 kDa). The same was observed with pure fibrinogen (10 mg/mL) solution, suggesting that hematin (30 μM) induces cross-linking of fibrinogen (Figure 4). Since there are no free thiol-groups in fibrinogen, the authors excluded cross-linking by disulfide bonding, but suggested heme-triggered dityrosine formation [237]. This was confirmed by fluorescence spectroscopy and ultra-performance liquid chromatography as well as by specific chemical modification of tyrosine residues, which completely abolished hematin-triggered cross-linking of fibrinogen [237]. Hou et al. (2018) confirmed these results of heme-induced fibrinogen cross-linking [238]. Again, direct hemin binding to fibrinogen was characterized by a red-shifted Soret band and a K_D of ~3.3 μM [238]. Additionally, a conformational change of fibrinogen (100 nM) with an introduction of an α-helical structure by hemin (25 μM) was observed by circular dichroism spectroscopy. One binding site within the γ-chain around the residues R^{256}, F^{293}, T^{371}, K^{373}, T^{374} and Y^{377} was suggested by applying an automatic docking tool [238]. Hemin (25 μM) binding to fibrinogen (50 nM) also increased

heme's peroxidase-like activity (ΔAbs ~0.17). Thus, the authors suggested the usage of this interaction for the label-free detection of fibrinogen in plasma samples [238].

In agreement with the fact that heme is capable of inducing cross-linking of fibrinogen by itself, just recently it was shown that heme (0.01–50 μM) does not have any impact on the amidolytic activity of the fibrin stabilizing active factor XIII (400 nM) [187].

Several researchers started to analyze the properties of the fibrinogen-heme complex. Therefore, there is no longer any doubt that heme binds to fibrinogen. However, the interaction remains not fully characterized. For instance, the binding affinity of heme towards fibrinogen or the actual heme-binding site(s) are still not known.

5.2. Heme Interaction with Factor VIIIa (FVIII(a)) and VWF

In 1967, Davis et al. discovered an inhibitory effect of hemin-derived hematoporphyrin on FVIII, which was attributed to a hematoporphyrin-driven destruction of FVIII [239].

About 15 years later, Glueck and colleagues observed reduced FVIII level upon infusion of 4 mg/kg hematin in an AIP patient [169]. Determined by a two-stage chromogenic assay [169], it can also indicate a reduced activity of FVIIIa instead of a decline of plasma concentration. Indeed, Green et al. (1983) demonstrated a concentration-dependent decrease of FVIII procoagulant activity in plasma in the presence of hematin (24 h aged; 18–70 μg/mL). The activity was reduced by up to 72% and 88% in pooled plasma and commercial FVIII concentrate, respectively [174].

These studies laid the foundation for an analysis of a potential direct interaction of hematin with FVIII. In 1986, Green et al. showed co-elution of the FVIII/VWF complex (36 U/mL of vWF:Ag) with hematin (0.17 mg/mL) after 30 min preincubation in SEC (Sepharose CL-4B column), suggesting binding of hematin to the procoagulant complex [240]. Indeed, the dissociation of FVIII from VWF was inhibited by hematin and binding of the FVIII/VWF-hematin complex to platelets was observed. The authors hypothesized that previous activation of platelets by hematin is required [240].

25 year later, apart from direct interactions, hemin (10 μM) was also reported to induce the formation of VWF fibers (ultra-large VWF) on the surface of endothelial cells as a consequence of Weibel Palade body (WPB) exocytosis [228]. VWF secretion by endothelial cells was later also observed by others, showing that it occurs in a TLR-4-dependent manner [26,206] (Figure 3). Due to the important role of VWF in platelet recruitment and clot formation this might be a crucial step in heme-triggered prothrombotic events.

Approximately at the same time, direct binding of heme in form of hemin was confirmed by UV/vis spectroscopy, revealing a Soret band shift to ~412 nm as well as rather high heme-binding affinities for the full-length (Helixate®; K_D ~12.7 nM) and the B-domain-deleted (ReFacto®; K_D ~1.9 nM) version of recombinant FVIII (rFVIII) [241]. Furthermore, a total number of ~10 heme-binding sites with heterogeneous affinity was estimated. The procoagulant activity of full-length (up to 50%) and B-domain-deleted (up to 51%) FVIII was impaired by heme (5 min preincubation) in a dose-dependent manner, as determined by routine factor X generation assay. In contrast to earlier reports [174], hematoporphyrin showed no impact on FVIII's procoagulant activity [241]. The procoagulant activity of FVIII is caused by its cofactor properties towards factor IXa and thus, the ability to support the generation of active factor X. Repessé and colleagues demonstrated that the interaction of rFVIII with FIX is impaired in the presence of heme (100-fold molar excess; by ~52%), thereby explaining the inhibition of FVIII's procoagulant activity [241]. In contrast, the interaction of FVIII with VWF, platelets and phosphatidyl serine was not altered. Interestingly, thrombin was still able to cleave FVIII when heme was present. VWF, but not albumin (up to 200-fold molar excess), protected FVIII from heme-driven inactivation. The authors thus concluded that VWF hides heme-binding sites rather than scavenging the heme [241].

So far, it is unclear whether or not the reported effects of heme on FVIII are relevant for in vivo situations. Although there is a massive release of labile heme under hemolytic conditions, it remains unclear whether consequently secreted VWF prevents from the

anticoagulant effect that was observed in case of FVIII in vitro. As one of the most important cofactors within the blood coagulation cascade, the inactivation of FVIII by heme could also play an important role as a central point for the control of heme-triggered prothrombotic effects (Figure 4).

5.3. Heme Interaction with Factor V (FV)

Glueck et al. (1983) recorded declined FV levels upon intravenous infusion of hematin (4 mg/kg) in an AIP patient [169]. Moreover, hematin was capable of reducing the activity of FV (by ~80%), as detected 10 min after hematin infusion (Supplementary Table S4; Figure 4) [169]. However, this is the only report on the effect of heme on FV to date and, thus, many details of a potential direct interaction are missing.

5.4. Heme Interaction with Factor XII (FXII)

After the observation of a decreased PTT (Section 3; Supplementary Table S2), Becker et al. (1985) searched for a potential effect of different porphyrins, including hematin, on the FXII-dependent pathway [176]. A dose-dependent increase of the amidolytic activity of the serine protease kallikrein towards a fluorogenic peptidic substrate (Bz-Pro-Phe-Arg-*p*-nitroanilide) was observed (e.g., in the presence of 12 nmol hematin, ~10-fold faster conversion), when human plasma was 10 min preincubated with hematin (3–24 nmol). When plasma was deficient of FXII, prekallikrein (inactive precursor of kallikrein) or high-molecular-weight kininogen (cofactor for kallikrein and FXII activation), hematin was unable to trigger the conversion of the fluorogenic substrate by kallikrein. Addition of a specific FXII inhibitor (CHFI) and the serine protease inhibitor SBTI completely abolished the hematin-mediated activation of kallikrein. Due to interference of hematin within the spectrophotometric measurements, the effect on the amidolytic activity of FXII towards a chromogenic substrate could not be analyzed. However, the authors demonstrated that PPIX was capable of increasing FXII amidolytic activity (78.6 µg/mL FXII, 38 µM PPIX) and autoactivation (9.7 µg/mL FXII, 1.9 µM hematin) [176]. The results clearly suggest a procoagulant effect of hematin on the FXII-dependent pathway of blood coagulation (Figure 4). Therefore, controversy to earlier reported anticoagulant effects of hematin were attributed to different preparations of hematin, since within this study hematin was freshly prepared in alkaline solution as the main difference to earlier studies [176]. However, blocking of the intrinsic, FXII-dependent pathway in vivo did not influence coagulation activation by heme (35 µmol/kg) in mice, which demonstrated that it is only dependent on the extrinsic, TF-driven pathway of coagulation [242] (Section 5.7). These results might refute the relevance of the in vitro results from Becker et al. (1985). Though, it is unclear to what extent heme would still bind to components of the FXII-dependent pathway in vivo under hemolytic conditions, while supporting its procoagulant effect on the extrinsic pathway of coagulation (Figure 4).

5.5. Heme Interaction with Thrombin

When Glueck et al. (1983) preincubated (0–180 min) thrombin (25 U) with hematin (70 µg/mL), clotting time was incubation time- and temperature-dependently prolonged, which led the authors to conclude a direct impact of hematin on thrombin [169]. A few years later, the generation of fibrinopeptide A resulting from the reaction of thrombin (0.25 U/mL) and fibrinogen (2.5 mg/mL) was radioimmunologically analyzed in the presence of four weeks aged hematin (6 µg/mL). Thrombin was preincubated (15 min) with hematin. As a consequence, fibrinopeptide A generation was ~100-fold blocked [243], which fits well to the investigations of Glueck et al. on thrombin and FDP in the presence of hematin [169] (Section 5.1). This also suggests that the resulting anticoagulant effect might be due to degraded hematin and not by hematin itself, as already shown for prolonged clotting times after hematin infusion [156,175] (Section 3). The same group suggested the possibility of direct binding of hematin to thrombin, after both co-eluted in SEC as also observed for fibrinogen (Section 5.1) [174]. Moreover, hematin was able to inhibit

the amidolytic activity of human α-thrombin towards a fluorogenic peptide substrate (D-Phe-Pro-Arg-5-aminoisophthalic acid dimethyl ester) by up to 86.3% (Supplementary Table S4) [174]. Once again, a 24 h old hematin solution was used, which might have influenced the results (as demonstrated later [156,157,175]).

More than 30 years later, Sparkenbaugh et al. (2015) detected elevated thrombin-antithrombin (TAT) level (up to 2.5-fold) upon retro-orbital injection of 35 µmol/kg heme in mice, which is a sign for coagulation activation [242]. In a time-dependent experiment, coagulation activation occurred 1 h after heme injection and was still increased 6 h after injection [242]. The authors demonstrated that this was not due to a direct interaction of heme with thrombin but caused by heme-induced TF expression (Section 5.7) [242].

In contrast to the studies of Green et al. from 1983 [174], hemin (0–50 µM) was recently shown to not influence the amidolytic activity of human α-thrombin (25 nM) [187] (Figure 4). While in the latest study a chromogenic peptide substrate (p-Glu-Pro-Arg-MNA) was used, in 1983 a fluorogenic substrate was added. Therefore, the earlier registered inhibitory effect could be caused by heme-associated fluorescence quenching and the results might be affected. However, the differences in heme preparation, buffer systems, and used substrates could also explain the different results.

5.6. Heme Interaction with Plasmin(Ogen)

For the first time in 1983, the effect of hematin on fibrinolysis was evaluated [174]. 15 µg/mL hematin (24 h kept in sodium carbonate buffer) was capable of inhibiting whole blood clot lysis. When hematin was first mixed with plasmin (0.017 U/mL) and then applied to the clot, the same result was obtained suggesting a direct, inhibitory effect of hematin on plasmin. Therefore, the impact of hematin (0.006–0.09 µg/mL) on the amidolytic activity of plasmin was analyzed. The ability of plasmin to cleave a fluorogenic peptide substrate (D-valine-leucine-lysine-5-aminoisophthalic acid dimethyl ester) was inhibited by 6–50% [174]. This was the very first report on a hematin-protein interaction that proposed a procoagulant effect of hematin. However, at the same time, plasminogen levels were shown not to be changed after infusion of hematin (4 mg/kg) in an AIP patient [169]. As already hypothesized for fibrinogen (Section 5.1) [234], also for plasmin and its inhibitor α2-antiplasmin a putative heme group was identified via LC-MS/MS, but has not yet been further investigated [244].

5.7. Heme Interaction with Adhesion Proteins

Apart from VWF, other adhesion proteins have also been reported to be upregulated by heme. For instance, already in 1997, Wagener and colleagues observed increased superficial expression of intercellular adhesion molecule 1 (ICAM-1; two-fold), vascular cell adhesion molecule 1 (VCAM-1; 3-fold), and E-selectin (4-fold) upon incubation of HUVECs with heme (50–100 µM, for 24 h) by using different techniques (e.g., dot blot immunoassay, ELISA) [245]. Later on, these observations were confirmed in mice that received a dose of 750 µM heme by intravenous infusion [220]. In particular, the surface of vascular endothelial cells in liver and pancreas were affected. While the expression of ICAM-1 and VCAM-1 was still increased after 24 h, P-selectin expression was only elevated 1 h after administration [220].

In 2009, Woollard et al. demonstrated that hemin even slightly induced endothelial collagen expression as demonstrated after perfusion of 1 mM hemin in mice aorta [204]. This might allow for the heme-triggered initiation of the intrinsic contact pathway of blood coagulation (Figure 4).

Moreover, hemin (10 µM) exposure of HUVECs resulted not only in an increase of WPB exocytosis but also of P-selectin expression on the surface of the cells within 5 min [228]. However, inhibition of TLR-4 completely blocked this effect, suggesting heme-induced P-selectin expression as a TLR-4 dependent process [228]. Then, Frimat et al. (2013) then confirmed the parallel expression of P-selectin on the surface of HUVECs and the exposure of VWF [26]. Due to the rapidity of expression, the authors suggested a correlation with

WPB mobilization [26]. Later, these observations were also made by others in vitro and in vivo (Supplementary Table S4) [206].

These results are of great importance, since these adhesion proteins enable the recruitment of different cell types to the endothelium. However, the molecular basis of the proposed interactions is still not entirely solved. Within the process of blood coagulation there are even more adhesion molecules involved, like the platelet endothelial cell adhesion molecule 1 (PECAM-1), but a regulation by heme was not yet explored. Thus, further investigation for a complete understanding of the role of adhesion proteins in heme-triggered coagulation is required.

5.8. Upregulation of TF by Heme

After several studies that reported the endothelial activation and adhesion molecule expression by heme, Setty et al. were the first who suggested a direct effect of heme on TF [246]. With different techniques, i.e., ELISA and flow cytometry, the authors found that heme (1–100 μM) dose-dependently increases TF expression (up to 50-fold) on the surface of micro- (HLMECs) and macrovascular (HUVECs) endothelial cells. Moreover, heme (100 μM) upregulated TF mRNA expression (up to ~17-fold) after incubation with endothelial cells. TF mRNA expression occurred slower and to a lower degree than cytokine-induced (e.g., by TNFα) [246]. Indeed, NF-κB activation inhibitors (i.e., sulfasalazine, curcumin) prevented from heme-triggered TF mRNA expression, suggesting a dependency of heme-induced TF mRNA expression from NF-κB activation. In line with a TF mRNA expression upregulation, total endothelial protein levels of functionally active TF were raised up to 20–39-fold after 4–7 h, tending towards a strong procoagulant effect [246].

A few years later Rehani et al. (2013) made the same observations in PBMCs and monocytes. 4 h incubation with 10 μM hemin yielded a ~40-fold and ~70-fold increase of TF activity, as determined by a one-stage clotting assay [247]. Hemopexin (15 μM) attenuated this effect. TF mRNA levels were also increased in monocytes (140–350-fold) after incubation with heme (2–4 h). Efficient impact of inhibitors led to the conclusion that heme might trigger TF expression via TLR-4, PKC, NADPH oxidase, and ERK-1/2 [247], as already shown for the expression of adhesion molecules on the surface of HUVECs [206] (Section 4.2).

In support of these studies, Souza and colleagues (2014) investigated heme-driven TF expression in PBMCs and neutrophils by applying different techniques (i.e., thromboelastometry, thrombin generation test) [248]. Heme (30 μM)-induced TF expression in PBMCs was confirmed, whereas no TF expression could be observed in neutrophils. In addition, the hypercoagulable state was characterized by a decreased coagulation time and time to maximal velocity [248].

Evidence for in vivo regulation of TF regulation by heme was provided by Sparkenbaugh and colleagues in 2015, who investigated TF-dependent coagulation activation in mice [242]. Indeed, inhibition of TF by an antibody abolished heme (35 μmol/kg)-driven coagulation activation in mice, as detected by TAT plasma levels. Subsequently, the authors demonstrated that 6 h incubation with heme (5–50 μM) induced TF expression as well increased TF activity in human PBMCs and RAW 264.7 mouse macrophages in combination with a dose-dependent increase of procoagulant activity. In contrast, heme did not induce TF expression in endothelial cells [242]. Further in vivo studies demonstrated that knock-out of TF in myeloid cells, hematopoietic cells or endothelial cells still allowed for heme-triggered coagulation activation. In mice with human TF expressed on hematopoietic cells and murine TF on non-hematopoietic cells, the authors observed that blocking of only both TF sources abolished heme-mediated coagulation activation. However, also in SCD mice elevated TAT level were recorded, which might confirm the previous observations that heme activates the coagulation cascade via the TF-dependent pathway. Again, these effects were completely prevented by hemopexin administration (280 μmol/kg) [242].

After De Souza and colleagues observed shortened clotting times upon addition of 30 μM heme to whole blood (Section 3), a TF-specific antibody was used to block its

activity. As a consequence, heme-induced coagulation was inhibited, thus, again providing evidence for the relevance of TF in heme's procoagulant role [186] (Figure 4).

The molecular basis for this interaction has not been entirely explored. Just recently, first insights were provided by a preprint from Hounkpe et al. (2020) [249]. Herein, heme (≥10 μM)-induced TF expression in PBMCs was shown as well as a dose-dependent (5–30 μM heme) increase of TF activity. Moreover, plasma mixed with heme (30 μM) for 4 h exhibit increased TF procoagulant activity, as detected by a one-stage clotting assay. Indeed, blocking of TLR-4 resulted in a complete inhibition of heme-triggered TF procoagulant activity. Thus, also this procoagulant effect of heme seems to be mediated through TLR-4 [249]. A second, very similar receptor for heme was suggested by others [250]. May et al. (2020) demonstrated direct heme binding (~2–3:1 heme:receptor; K_D ~6.78 μM) to the receptor for advanced glycation end products (RAGE), which resulted in receptor oligomerization [250]. Thereby, signaling of heme via RAGE was followed by the phosphorylation of ERK-1/2 and Akt, as also earlier shown by others [221,222,247]. Experiments in wild-type and RAGE knockout mice suggest an involvement of RAGE in heme-triggered TF expression, since TF expression was less in RAGE knockout mice [250].

However, more investigation is required to unravel the whole network of heme-induced signaling pathways that lead for example to elevated TF expression, and, thus, to prothrombotic states.

5.9. Heme Interaction with Anticoagulant Proteins

To date, the effect of heme on the anticoagulant pathways is largely unknown. Antithrombin-III levels were shown to be influenced neither by the injection of hematin (4 mg/kg) in AIP patients [169] nor by the infusion of 3 mg/kg heme arginate (Normosang®) in healthy volunteers [177]. In contrast, in CLP mice administration of hemin (50 μmol/kg) clearly resulted in an upregulation of protein C and APC plasma levels as well as in a decrease of thrombomodulin plasma levels [184]. These effects that tend to an anticoagulant action were attributed to the role of hemin as an inducer of HO-1 [184].

In 2020, transient heme binding to APC was demonstrated for the first time [187]. This binding was characterized by a K_D of ~400 nM. Moreover, experimental studies allowed for the assumption of two heme-binding sites that subsequently were identified within the serine protease domain (heavy chain) of the enzyme (i.e., T^{285}GWGYHSSR^{293}, W^{387}IHGHIRDK^{395}) with Y^{289} and H^{391} serving as the coordinating residues [187]. A molecular dynamics simulation suggested a conformational change upon binding of two heme molecules, as seen by a more rigid light chain of the protein. Furthermore, in complex with heme (0.01–50 μM), plasma-derived APC (50 nM) was inhibited in its amidolytic activity with an IC_{50} of ~10.41 μM and a K_i of ~12.56 μM. For the recombinant APC (Drotrecogin alfa, Xigris ®) an inhibition by heme (IC_{50} of ~3.88 μM) was also observed [187]. As already observed for the fibrinogen-heme complex (Section 5.1; [238]), the APC-heme complex exhibits an increased peroxidase-like activity (~512.33% in comparison to 100% by heme only), as well. Moreover, heme (≥ 10 μM) abolished the anticoagulant activity of APC (5 nM) as demonstrated with an aPTT-based clotting assay, displaying a procoagulant role of heme. Indeed, human serum albumin (0.1%) could protect APC up to a certain extent. However, under these conditions, again, 100 μM heme was able to inhibit the anticoagulant activity of APC. Interestingly, the cytoprotective function of APC was not affected by heme. Quite the contrary, APC (20 nM) was capable of preventing heme (120 μM)-induced hyperpermeability of HUVECs, and, thus, protecting from heme-driven cytotoxicity [187]. Thus, this was the first detailed report on a direct interaction of heme with one of the endogenous clotting inhibitors (Supplementary Table S4). Although this effect of heme has not been proven in vivo so far, the interaction of heme with APC seems to be versatile. Beyond the formation of a peroxidase-like complex, and direct protection of endothelial cells from heme-directed loss of permeability by APC, heme inhibits the anticoagulant function of APC, which results in the support of procoagulant reactions.

Beside direct upregulation of procoagulant factor and the promotion of their procoagulant activities, heme, thus, is also capable of the inhibition of an inhibitor of the blood coagulation cascade, that usually targets FVa and FVIIIa (Supplementary Table S4, Figure 4).

6. Conclusions

This review presents labile heme as a multifaceted molecule with versatile effects in the context of blood coagulation. Extensive studies over the past ~110 years described the consequences of the infusion of heme and its different formulations in in vivo studies and case reports, as well as the impact of heme on different cell types and proteins that participate in the coagulation process.

Originally, Pierach and Rosborough suggested both an anti- and a procoagulant role of heme [251]. In early studies, heme (as hemin or hematin) was infused in rather high concentrations (10–180 mg/kg; single exception: ≥3.5 mg/kg) into animals resulting in symptoms of bleeding, i.e., hemorrhage, ecchymosis, (internal) bleeding, and hematomas (Figure 1). In contrast, in humans, healthy volunteers or AIP patients, lower amounts of heme (1.2–6 mg/kg) were administered (as hematin or heme arginate), which commonly caused thrombotic complications, including thrombophlebitis and bile thrombi (Figure 1). Opposed to these observations, but in line with the animal studies, heme (primarily used in the form of hematin) caused prolonged aPTT, PT, and TT (Figure 2). To date, however, it is unclear to which extent the anticoagulant symptoms were caused by heme itself. Several studies demonstrated that long-storage of heme in solution might lead to the generation of oxidative degradation products that are responsible for the observed effects [156,157,175]. In more recent studies, freshly prepared heme had either no or a shortening effect towards clotting times, suggesting a rather procoagulant role.

Accordingly, several cell types participating in blood coagulation, including platelets, RBCs, endothelial cells, and leukocytes, were shown to be affected by heme in a prothrombotic manner (Figure 3). Heme-induced hemolysis of RBCs as a feedback mechanism was not yet directly considered in the context of heme-triggered prothrombotic consequences. This process might lead to a potentiation of the observed effects due to the release of even more labile heme into the vascular compartment. Through heme-induced conformational changes of RBC membrane proteins the RBC membrane stability can be reduced. Furthermore, heme can accumulate in RBCs' membrane. Thus, RBC membrane microparticles are able to transfer heme to the endothelium, which can, in turn, activate endothelial cells. Indeed, heme infusion was associated with direct effects on the endothelium, such as the ACH-dependent vasodilation. Activation of TLR-4 by heme triggers the exocytosis of WPBs, which results for example in the release of VWF [26,206,228] (Figure 3). Furthermore, the expression of adhesion proteins is upregulated by heme in a TLR-4 and ROS-dependent fashion [206,208]. The exposure of VWF and different adhesion proteins, such as P-selectin and VCAM-1, enables the adhesion of platelets and leukocytes to the endothelium, promoting procoagulant processes. As the heme-driven endothelial activation, also the prothrombotic processing of leukocytes is mediated via TLR-4. Activation of TLR-4 by heme again triggers the adhesion of leukocytes to the endothelium [206]. In addition, neutrophils might respond with the formation of NETs upon heme exposure, which is mediated via the NADPH oxidase and consequent ROS generation [227] (Figure 3). Again, adhesion of platelets and RBCs is facilitated. Apart from these signaling pathways, also necroptotic and apoptotic pathways may play a role in heme-triggered endothelium activation [208,209].

Heme-triggered denudation of endothelial cells along with an exposure of collagen has been shown to be necessary for heme-associated platelet adhesion and consequent aggregation. Overall, two main mechanisms have been described for the platelet activation by heme. Direct heme binding to C-type lectin-like receptor 2 (CLEC2) induces a tyrosine kinase network that leads finally to the activation of platelets [199]. In addition, heme-induced ROS generation can promote the expression of adhesion proteins, that, in

turn, enable the adhesion and subsequent activation of platelets [196,198]. In contrast to endothelial cells, for platelets, the relevance of ferroptotic signaling has been shown [198].

Leukocyte accumulation in various organs, such as the liver and spleen, was observed as a consequence of heme exposure [220]. As already described for the endothelium activation by heme, heme-induced leukocyte rolling and adhesion also occurred TLR-4-dependently [206]. Moreover, heme-triggered neutrophil migration and recruitment were accompanied by PKC activation, oxidative stress, and actin polymerization [221,222]. However, heme-induced NET formation might be an essential step, since NETs are not only able to promote proinflammatory signaling but can also stabilize blood clots [224,252].

Finally, within the last few years, a remarkable development towards the investigation of heme as a mediator of protein expression and activity could be observed (Figure 4). In particular, the procoagulant proteins TF, fibrinogen, and VWF seem to be essential key targets that confer heme a procoagulant character (Figure 4). The inhibition of the anticoagulant activity of APC clearly supports this assumption. In contrast, the interaction of heme with FVIII(a) tends to an anticoagulant character of heme. This can be prevented by VWF, suggesting an inferior role of the proposed interaction or a counteracting effect together with the interaction of heme on FV. Since FV and FVIII are the central cofactors within the blood coagulation cascade, their interaction with heme might serve as a control mechanism that prevents from further potentiation of thrombosis at a certain state.

A potential interference of heme with several other proteins of the coagulation process, such as factor IX or factor X, was not yet examined. Furthermore, heme-binding affinities are only known for fibrinogen, FVIII(a) and APC. In the future, the characterization of the heme-binding capacity of other clotting factors and/or inhibitors might complete the whole picture of the range of the role of heme as an effector molecule in the blood coagulation cascade. However, due to its interference with different components at various steps of the coagulation system, it is already obvious that heme cannot only trigger coagulation activation, in particular by effects on endothelial cells and platelets as well as by the upregulation of relevant factors (e.g., TF and VWF), but can also promote further clotting through initiation, amplification, and propagation of plasmatic hemostasis.

This review provides a comprehensive overview of the broad range of heme's actions as a modulator of blood coagulation, thereby in particular emphasizing its relevance in hemolysis-driven thrombosis. A role of heme in the development of thrombotic complications in SCD has in particular been proposed [18,253].

Furthermore, the detailed insights summarized herein regarding the molecular basis of heme-triggered coagulation allow for the targeted investigation of the missing interrelations and as yet unknown potentially regulatory heme–protein interactions. Finally, the actual role of heme in the coagulation system and as a promoting factor for prothrombotic events in hemolytic patients depends on the availability and quantity of heme and plasmatic proteins, including clotting factors, anticoagulant/fibrinolytic proteins and adhesion proteins, the heme-binding affinity of those proteins which directly bind heme, the location of relevant cell types with respective target receptors, such as TLR-4, and the chronology of events. An in-depth analysis of these factors might help to entirely understand the molecular basis of heme-triggered thrombosis in hemolytic disorders and support the development of suitable therapies within the future. Overall, due to the conspicuous clinical manifestation of thrombophilic reactions upon hemolysis, heme should be considered in the treatment of these complications, rather than focusing on a sole treatment of thrombotic symptoms [17,20].

Supplementary Materials: The following are available online at https://www.mdpi.com/2077-0383/10/3/427/s1, Table S1: Overview of side effects observed after heme injection, Table S2: Overview of heme effects on bleeding and clotting times, Table S3: Overview of the impact of heme intoxication on cells participating in blood coagulation, Table S4: Overview of the impact of heme and its formulations on proteins acting in blood coagulation.

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4.1.2 Summary

Approximately 110 years of research characterizes heme as an actor in blood coagulation. Initially, its role, may it be anticoagulant or procoagulant, has been controversially discussed. The comprehensive compilation of these data allows for the conclusion that rather oxidative heme degradation products than heme itself tend towards an anticoagulant property. Furthermore, high doses of heme might promote hemorrhage, which has only been shown in animals. In patients with acute intermittent porphyria heme infusion induced slight thrombophlebitic reactions as a result of locally increased heme levels. The current available heme-based drugs Panhematin® and Normosang® seems to have less risk for coagulation abnormalities due to the respective formulation of heme (either with sorbitol or arginate, respectively). More importantly, effects of heme on cells that participate in the blood coagulation process have been demonstrated and analyzed on the molecular basis *in vitro* and *in vivo*, evidencing the induction of prothrombotic reactions in hemolytic disorders. A major role for the heme-promoted activation of cells in blood coagulation plays the TLR4 signaling pathway, as it causes TF expression in leukocytes, WPB exocytosis in endothelial cells and α-granule release of platelets. The importance of other receptors, CLEC2 and RAGE, was recently suggested. Furthermore, heme-induced ROS production plays a crucial role, which leads to the activation of apoptotic signaling pathways in endothelial cells and NET formation in neutrophils. In total, these processes trigger platelet aggregation and the initiation of secondary hemostasis. The latter is directly affected by heme as well. While the heme-driven activation of FXII, plasma kallikrein and fibrinogen leads to the propagation of procoagulant reactions, the inactivation of FVIII tends to anticoagulant effects. Nevertheless, the relevance of the FVIII-heme interaction has not been deciphered yet. However, direct heme binding and affinity has only been characterized for fibrinogen and factor VIII.

Hence, this review article emphasizes the need for further investigation of the interactions of heme within the blood coagulation cascade on the molecular basis. Although the impact of heme on cellular components is largely known, the actual triggered signaling pathways are not well characterized and only a few effector molecules have been identified yet. So far, just a few blood coagulation proteins were analyzed for their heme-binding capacity. Both the missing information on potential heme-protein interactions as well as on heme-induced signaling pathways in heme-induced thrombosis form the basis for the following studies (cf. Chapter 4.2 and 4.3, respectively).

4.2 Molecular insights and functional consequences of the interaction of heme with activated protein C

Original research article

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*__Contribution:__ M.-T.H., N.S.H., B.P., and D.I. conceived and designed the study. M.-T.H. performed the experiments and collected the data. N.S.H. performed the barrier protection assay. N.A., A.A.P.G., and A.B. carried out the computational studies. M.-T.H., N.S.H., B.P., A.A.P.G., A.B., and D.I. analyzed the data. B.P. and J.O. contributed analysis tools. The article was written through the contributions of all authors.

4.2.1 Introduction

In the past, several heme-regulated proteins were identified and characterized, displaying the versatile role of heme in various biochemical processes, such as signal transduction, transcription, and inflammation.[10,424] However, in the blood coagulation system, only fibrinogen and FVIII were identified as heme-binding proteins.[20,21,413] Based on earlier established criteria and by use of the application HeMoQuest,[423,426–431,435] the anticoagulant serine protease APC was predicted as a potentially heme-regulated protein. APC is a multifunctional enzyme with antiinflammatory, cytoprotective, profibrinolytic, and anticoagulant properties,[312,313] presenting an attractive target for heme binding. Thus, within the following publication, for the first time an inhibitor of the blood coagulation system, i.e. APC, is fully characterized as a heme-regulated protein. Despite of the analysis of direct heme binding, functional assays in the presence of heme allow for the assumption of a (patho-)physiological role of heme binding to APC.

Original Research Communication

Molecular insights and functional consequences of the interaction of heme with activated protein C

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Abstract

Aims: In hemolysis, which is accompanied by increased levels of labile redox active heme and often associated with hemostatic abnormalities, a decreased activity of activated protein C (APC) is routinely detected. APC is a versatile enzyme that exerts its anticoagulant function through inactivation of clotting factors Va and VIIIa. APC has not been demonstrated to be affected by heme as described for other clotting factors and thus is subject of investigation.

Results: We report the interaction of heme with APC and its impact on the protein function by employing spectroscopic and physiologically relevant methods. Binding of heme to APC results in inhibition of its amidolytic and anticoagulant activity, increase of the peroxidase-like activity of heme and protection of HUVECs from heme-induced hyperpermeability. To define the sites that are responsible for heme binding, we mapped the surface of APC for potential heme-binding motifs. T^{285}GWGYHSSR^{293} and W^{387}IHGHIRDK^{395}, both located on the basic exosite, turned out as potential heme-binding sites. Molecular docking employing a homology model of full-length APC indicated Tyr^{289} and His^{391} as the Fe(III)-coordinating amino acids.

Innovation: The results strongly suggest that hemolysis-derived heme may directly influence the protein C pathway through binding to APC, conceivably explaining the decreased activity of APC under hemolytic conditions. Furthermore, these results extend our understanding of heme as a multifaceted effector molecule within coagulation and may allow for an improved understanding of disease development in hemostasis under hemolytic conditions.

Conclusion: Our study identifies APC as a heme-binding protein and provides insights into the functional consequences.

Introduction

The redox active molecule heme is known for its essential role as the prosthetic group in a number of hemoproteins, such as hemoglobin and cytochromes. Each of up to 25 trillion circulating red blood cells (RBC) in the human body is thought to contain 2.5 x 10^8 hemoglobin molecules with 5 x 10^9 molecules of heme bound, which roughly corresponds to 80 mM heme (9, 10). Under hemolytic conditions, heme is released as a result of erythrocyte lysis and subsequent degradation of hemoglobin, which in turn may lead to repletion of the heme scavenger hemopexin (57). Heme may also adhere to other plasma proteins, such as albumin and α1-microglobulin, resulting in a pool of so-called “labile heme” (63). Labile heme is well-known as a regulator of various proteins in a broad range of biochemical and pathophysiological processes, such as transcription, signal transduction, inflammation or hemostasis, but it is still unresolved to which extent it might influence coagulation processes, e.g., in hemolytic states (19, 37, 57).

Intravascular hemolysis has been reported to increase the thrombotic risk (1, 44). This can be partially explained by hemolysis-induced coagulation activation through exposure of negatively charged phospholipids from ruptured RBCs, induction of tissue factor expression, and stimulation of von Willebrand factor release from endothelial cells (37, 57). However, the underlying mechanisms of these events have not yet been elucidated (1, 16, 54).

Interference of heme with endogenous inhibiting systems such as the protein C (PC) pathway, might contribute to the hemolysis-induced hypercoagulable state. PC is the zymogen of the serine protease activated PC (APC) that is synthesized in a vitamin K-dependent manner in the liver and circulates at a concentration of 70 nM in human blood (23). PC consists of a heavy and a light chain connected by a disulfide bond (32). The light chain is composed of a Gla domain and two epidermal growth factor (EGF)-like domains, whereas the heavy chain comprises the serine protease domain. Depending on the glycosylation pattern of the heavy chain, different molecular forms (α, β, and γ) of protein C exist (20). PC becomes activated to APC through limited proteolysis and cleavage of the activation peptide by the thrombin-thrombomodulin complex on the luminal surface of endothelial cells. Once formed, APC down-

regulates further thrombin formation by proteolytic inactivation of the procoagulant cofactors activated factor VIII (FVIIIa) and activated factor V (FVa) (56). In addition, APC can exert pleiotropic cytoprotective effects, including the activation of antiapoptotic and antiinflammatory, pathways enhancing e.g. endothelial barrier integrity. These effects are induced by APC-mediated activation of the G-protein coupled protease-activated receptors 1/3 (PAR1/3) and involve binding of APC to the endothelial protein C receptor (EPCR) (21).

The increased thrombotic risk of inherited PC deficiency, inherited PS deficiency, and of APC resistance emphasize the importance of this anticoagulant pathway (3, 43, 66). In addition, Gabre *et al.* reported that APC can induce the expression of heme oxygenase 1 (HO-1), an enzyme that catalyzes the degradation of heme (17). Earlier, however, an upregulation of PC and thrombomodulin by HO-1 was shown by Fei and coworkers (13). Continuing the line, in patients with sickle cell disease (SCD) accompanied by severe hemolysis, decreased levels of APC were partially reported based on the amidolytic activity of the enzyme, however, without describing the underlying mechanism (60, 67).

In previous studies we identified potential heme-regulated proteins such as interleukin-36α and cystathionine-β-synthase, which were unknown to bind heme (6, 39, 49, 61, 70). Herein we provide evidence that human APC is a heme-binding protein and thus might represent a heme-regulated protein, as well. Surface plasmon resonance (SPR) spectroscopy and *in silico* studies were used to characterize the respective interaction. Potential heme-binding sites on the surface of APC were identified and analyzed by UV/vis spectroscopy. In order to visualize heme binding to APC we employed molecular modeling and docking studies using an in-house generated homology model. Moreover, we demonstrated an impact of heme on the amidolytic and anticoagulant function of APC and observed an increased peroxidase-like activity of the heme-APC complex. Apart from that, the cytoprotective functions of APC were not influenced by heme binding as human umbilical vein endothelial cells (HUVECs) were protected from heme-induced cytotoxicity in the presence of APC. Taken together, our data provide novel valuable insights into the regulation of the anticoagulant protein APC by heme, which may

contribute to our understanding of the physiological functions of APC as well as the development of hemolytic diseases associated with hemostatic abnormalities.

Results

Heme binds to human APC

In order to demonstrate and characterize the binding of heme to APC, SPR measurements of the heme-APC interaction were performed. The analysis of the association of heme with APC using a global 1:1 kinetic model revealed a K_D value in the nanomolar range (400 ± 47 nM) according to the best fit (Figure 1). The binding of heme to APC is transient, which is characterized by a rapid association (k_a = 2.06 x 10^4 $m^{-1}s^{-1}$) and dissociation process (k_d = 8.08 x 10^{-3} s^{-1}). A stoichiometry of approximately 1:1.3 (protein:heme) was determined, which indicates either overlaying binding events of heme to different binding sites, nonspecific binding or both.

Heme binds to APC-derived peptides

Previously it was shown that nonapeptides represent suitable models for analyzing heme-regulatory/binding motifs (HRM/HBM) on the surface of proteins (39, 40, 49). Four APC-derived nonapeptides were included in this study (peptides **1** – **4**, Table S1), which were predicted by the in-house developed webserver HeMoQuest (48) based on earlier established criteria for heme binding to proteins (5, 7, 38, 39). These peptides, containing either histidine and/or tyrosine as the coordinating residue, were synthesized and analyzed for their heme-binding capacity by UV/vis spectroscopy. Heme binding was observed by a Soret band shift to ~ 420 nm for all peptides (Figure 2, Figure S1). It was previously demonstrated by different spectroscopic methods, including rRaman, *cw*EPR and 2D-NMR spectroscopy that a shift to ~ 420 nm is typical for a mixture of a hexa- and pentacoordination or hexacoordination of a heme-peptide/protein complex (5, 7, 39, 61). According to the best fit, a stoichiometry of 1:1 was determined for all heme-peptide complexes investigated herein. The highest heme-binding affinities were observed for peptides **1** (TGWG**YH**SSR) and **2** (WI**H**G**H**IRDK) displaying K_D values of 0.46 ± 0.25 µM and 1.65 ± 0.51 µM, respectively. The sequence stretch of peptide **1** is located close to the active site of APC and found within a coiled structure (Figure

2A-C). In contrast, the motif of peptide **2** is part of a helix, further away of the active site and C-terminal of the heavy chain, but within the basic exosite of the protein (Figure 2A-B, D). Two further potential motifs (peptides **3** and **4**, Table S1) revealed only moderate affinities for heme and, in contrast to **1** and **2**, they are distant from the active site. The motifs **1** and **2** thus represented promising candidates for potential heme-binding sites in APC.

In silico study of heme-APC complex via molecular docking and MD simulations

In silico structural modeling of APC was employed in this study because an experimentally derived structure of the full-length APC is not available so far (45). Earlier reported homology models of APC were performed for either the serine protease domain or the zymogen form of protein C (14, 51). In our work, the five top-ranked templates used to build the initial homology models of APC were PDB ID: 1AUT, 3F6U, 2AER, 1W0Y, 3HPT, and had an alignment target coverage of 83%, 83%, 94%, 93%, and 80%, respectively. The final hybrid model (Figure 2A) consisted of 405 residues (full coverage of APC(1-405), i.e. without activation peptide) and included contributions from the following initial models: 1AUT-~(39-393), 1W0Y-~05(1-52), 1W0Y-~02(68-76), 2AER-~01(394-406) (Table S2). To evaluate the quality of the model, Z-scores, which indicate the total deviation in energy against energy distributions derived from entirely random conformations, were used as a metric. In an ideal situation, a value of 0 is expected but in most real-world cases values close to 0 are acceptable (68). Accordingly, the overall Z-score~~,~~ obtained as a weighted average of the individual Z-scores of three distinct components namely, dihedrals, 1D packing and 3D packing, was found to be -1.407. Ramachandran plot analysis of the structure showed that 87.2% of the models' residues were in the most favored regions, 12.2% of residues in the allowed region and a meagre 0.6% of the residues in the disallowed region indicating that the final model obtained was of good quality. As a result of focused molecular docking experiments on the HRMs **1** – **4**, clusters of docking complexes were automatically ranked and sorted by the Autodock Vina scoring function used (65). Each cluster was then manually inspected to select only those complexes that had coordination interactions between potential HRMs and a surface-bound heme

molecule (based on a distance cutoff of 3 Å) that suggests transient heme binding. Our analysis revealed that two (motifs **1** and **2**) out the 4 investigated motifs exhibited an excellent heme-binding potential based on favorable heme-motif distances and the relative strength of their association quantified by Poisson-Boltzmann binding energies. The stability of the heme-motif interaction for the motifs **1** and **2** was evaluated based on the observed residence of heme at the motif in APC through the entire course of the 50 ns simulation runs, quantified by the distances between the Fe^{3+} ion of heme and the side chain oxygen of the Tyr^{289} residue (motif **1**) and the side chain nitrogen atom of the coordinating His^{391} residues (motif **2**), respectively (Table S3). For motif **1** this average distance (between Tyr^{289} and the Fe^{3+} ion of heme) was 2.16 Å and the Poisson-Boltzmann binding energy (see Materials and Methods) estimated to quantify the heme-motif association was -168.08 kJ/mol. Motif **2** displayed similar positive binding characteristics as the distance between the His^{391} residue of the motif was at an average of 2.07 Å and a consequent binding energy of -255.49 kJ/mol. These observations serve as corroboration to the experimental results at the peptide level. Based on the distances discussed herein, though it might be the case that both the motifs **1** and **2** stand a similar chance and potential of heme coordination, the binding energy analysis suggests that motif **1** with a more positive value (-168.08 kJ/mol) might have a higher preference over motif **2** (-255.49 kJ/mol), since the binding energy quantify the strength of the association of heme to its respective motifs. On the other hand, observations from motifs **3** and **4** showed clear indications of poorer heme binding capabilities (Table S3, Figure S2). Motif **3**, despite holding heme at an average distance of 2.18 Å away from the motif had a much lower binding energy of -326.51 kJ/mol. The average heme-motif distance among all the four investigated motifs was the highest for motif **4** with at 4.03 Å an associated binding energy of -291.95 kJ/mol.

With the individual motifs investigated, a 50 ns MD simulation of heme bound to both motif **1** and **2** showed that both the heme molecules stayed bound to the surface of APC with heme-motif distances under ~ 3 Å over the course of the simulation (Figure 3A, Video S1). This suggests that APC has the capacity to bind more than one heme molecule and in theory can bind up to two heme molecules at a time.

The structural consequences of heme binding to APC was assessed via two separate 300 ns long MD simulations conducted on both the heme-bound and unbound form of the protein. We observed that the association of the two heme molecules with APC had a marked impact on the dynamics of APC by inducing a significant reduction of the inherent flexibility/mobility of the protein. The per residue root mean square fluctuation (RMSF) of the residues was used as a MD-derived measure to compare the dynamics of the heme-bound and unbound form of APC. The RMSF values of the two simulations clearly indicate that heme binding to APC has a dampening effect on the natural dynamics of the protein (Figure 3B). This structural impact can be hypothesized to have a functional consequence on the protein.

Heme inhibits the amidolytic activity of APC

The amidolytic activity of APC was analyzed in the presence of different concentrations of p-Glu-Pro-Arg-MNA as the substrate, revealing a K_M value of 0.42 ± 0.07 mM, which is in the same range as reported for other chromogenic peptide substrates of APC (Figure S3) (59). In the presence of heme, the inhibition of the APC activity by heme as a competitive inhibitor revealed a K_i value of 12.56 ± 2.31 µM (Figure 4A). In comparison, the amidolytic activity of APC was also studied in the presence of two other porphyrin analogs, namely zinc protoporphyrin IX (ZnPPIX), and protoporphyrin IX (PPIX) as well as iron(III) chloride at concentrations ranging from 0 – 50 µM. Heme inhibited the activity of plasma-derived APC (pdAPC) and recombinant APC (rAPC) in a dose-dependent manner with an IC_{50} value of 10.41 ± 1.24 µM and 3.88 ± 0.87 µM, respectively, according to the best fit (Figure 4B, C). At the highest concentration of heme (50 µM) applied, an inhibition of 80% of pdAPC's activity was observed. A Hill slope of 2.0 suggests two binding sites for heme with positive cooperativity (n = 2). A similar effect towards pdAPC was observed after incubation with protoporphyrin IX, revealing an IC_{50} value of 8.46 ± 1.68 µM, n = 2 (Figure 4D). Zinc protoporphyrin IX had an even stronger effect by decreasing the pdAPC activity to 1.42% (IC_{50} = 4.33 ± 0.87 µM, n = 2, Figure 4E), which has been shown for zinc(II) ions as well by Zhu *et al.* (77). Iron (III) ions reduced the pdAPC activity only to 66.83% at a concentration of 50 µM

(Figure 4F). To compare the effect of heme on APC's activity with that on other enzymes of the blood coagulation cascade, the enzymatic activities of the serine protease thrombin and the transglutaminase factor XIIIa (FXIIIa) were observed in the presence of heme. Both, thrombin and FXIIIa, were not affected by heme in their enzymatic activities (Figure 4G, H). Indeed, these proteins were not detected as heme-binding proteins with the publicly available webserver HeMoQuest (48) indicating reliability of the program and the underlying search criteria concerning heme-binding motifs.

Heme-APC complex shows peroxidase-like activity

The peroxidase-like activity of heme-protein complexes has been reported earlier with amyloid beta being the most prominent protein representative evoking a significantly increased activity (~500%) compared to uncomplexed heme (2, 71). The activity of the heme-APC complex was determined using an experimental set-up earlier established (2, 64, 71). Normalized against the basal heme activity, the heme-APC complex showed a more than 5-fold higher activity (512.33 ± 66.50%; Figure 4I) and thus, is one of the most efficient molecules regarding this effect compared to the peptides and proteins which have been studied so far (28, 29, 64, 71).

Heme is able to inhibit the anticoagulant activity of APC

To investigate the impact of heme on the anticoagulant function of APC, the conventional activated partial thromboplastin time (aPTT)-based clotting assay was performed with plasma samples. Heme alone had no significant effect on aPPT, whereas APC (5 nM) prolonged the clotting time of normal plasma by ~2-fold (Figure 5A). An incubation of APC with 1 μM heme did not change this effect. In contrast, the preincubation of APC with heme at 10 μM and higher concentrations caused a marked reduction of the clotting time, counteracting the anticoagulant function of APC. Thus, the clotting time was effectively shortened to that of normal plasma without addition of APC. Upon preincubation with heme (up to 50 μM), the addition of exogenous albumin (0.1%) completely restored the clotting time to the level when only APC was applied (Figure 5B). At 100 μM heme, however, a tendency towards inhibition of the

anticoagulant function of APC was observed again (not statistically significant). Due to the solubility and high aggregation potential of heme in correlation with the required assay conditions, the effect of higher concentration could not be investigated despite of the pathological relevance. Further studies demonstrated that heme could already induce a slight inhibition of APC's anticoagulant without any preincubation, which is more emphasized after a preincubation time of 15 min or longer (Figure S4).

Since the heme analogs ZnPPIX and PPIX were potent to inhibit the amidolytic activity of APC similar to heme, their impact on the anticoagulant function was investigated as well. Like heme, none of the analogs had a direct influence on aPTT. However, after pre-incubation with APC and the addition of this mixture to plasma, aPTT was shortened (Figure 5C, D). In the presence of additional HSA, an effect comparable to heme was observed (Figure 5D). The lowest concentration of heme still exerts activity in the absence of HSA (i.e. 10 µM) seemed to be not sufficient for the heme analogs (Figure 5C). In order to achieve the same effect as heme in this approach, an increase of their concentration to 100 µM was required (Figure S5).

APC protects HUVECs from heme-mediated hyperpermeability

To study the influence of heme on the cytoprotective function of APC, an endothelial barrier protection assay with HUVECs was performed. Heme exposure of the HUVECs resulted in a concentration-dependent increase of FITC-dextran flux through the cell monolayer (Figure S6). In the presence of APC, the HUVECs were protected from heme-induced hyperpermeability. Therefore, the cytoprotective function of APC was not influenced by heme (Figure 6).

Discussion

The present study provides evidence that heme binds to APC and thereby impacts its function (Figure 7). We demonstrate a heme-binding affinity for APC in the nanomolar range, which is typically found for heme-regulated proteins, as can be exemplified with the transcription repressor Bach1 (K_D = 140 nM) or heme oxygenase 2 (K_D = 33 nM (dithiol state), K_D = 350 nM (disulfide state)) (47, 76). The transient manner of heme binding to APC is illustrated by both, rapid association and dissociation of the heme molecule. A stoichiometry of 1:1.3 – 1:2 suggested one to two binding sites for heme with either overlaying binding events, different binding affinities of the two motifs or a mixture of specific and unspecific binding events. Plasma-derived APC represents a mixture of different glycosylated forms of APC (α, β, and γ). Different accessibility of potential binding sites through a missing glycosylation may result in different binding properties, which, in turn, may potentially affect the stoichiometry. This is further evidenced by the computational studies using a combination of molecular docking and MD simulations suggesting heme binding to both motifs **1** and **2** with a comparably high potency. We identified T^{285}GWG**Y**HSSR^{293} (motif **1**) as the most probable heme-binding site on the surface of APC with Tyr^{289} as the favorable coordination site and the highest affinity compared to other motifs. This motif is located close to the active site, which might explain the effect of heme on the amidolytic activity of APC, accomplished by either disturbing access to the active site or a conformational change of the protein infringing the integrity of the active site (Figure 8). Based on experimental and MD simulation results we suspect that W^{387}IHG**H**IRDK^{395} (motif **2**) with His^{391} as the coordinating residue is one further high affinity heme-binding site that could be responsible for overlaying binding effects, which were observed in the SPR studies or represent the second binding site as determined with the amidolytic activity assay in presence of heme (Figure 8). Molecular docking studies allowed for the assumption that heme binding to both motifs is possible, which fits to the aforementioned results. Moreover, two other motifs showed moderate heme-binding properties based on experimental and computational approaches. Thereby a recruiting binding process could be conceivable. The proposed binding sites (motif **1** and **2**) were already described as

potential binding sites for zinc ions of APC (77). Moreover, the sequence of motif **2** was earlier reported to be part of a sequence stretch that is crucial for the anticoagulant function of human APC (46). The potential coordinating site of motif **1** is highly conserved in APC from different species, whereas motif **2** is missing in some species, but, is also highly conserved, if present (Table S4). However, both motifs are part of the basic exosite, which is essential for the interaction of APC with FVa and FVIIIa and proper presentation of both factors to the active center (Figure 7B, C) (56). Heme binding to APC may thus impair the catalytic activity of the protein and its interaction with other coagulation factors. Our MD-based observations indicate that the heme-induced conformational change could be initiated by a reduction of the protein flexibility. However, a clear characterization of the conformation change itself is not within the scope of this study as this might happen at a much larger simulation time scale than 300 ns due to the size of the system (>250,000 atoms) and is solely hypothetical.

However, heme is indeed able to inhibit the amidolytic activity of rAPC and pdAPC with an IC_{50} value of 3.88 ± 0.87 µM and 10.41 ± 1.24 µM, respectively. The recombinant form merely differs from the plasma-derived APC in the glycosylation pattern (75). While the recombinant form often shows a higher content of the highly active β-APC (diglycosylated heavy chain) and only 50–60% of the α-form (triglycosylated heavy chain)), the pdAPC contains 70–80% of α-APC (22). In addition, the oligosaccharides in rAPC are described to be less branched, since they are only diantennary (74). Thus, the higher impact of heme on the recombinant form might be due to less hidden binding sites and an improved access of heme to these sites. Nevertheless, the fact that heme is able to impair the conversion of a small peptide substrate by APC might be an indication for the lower APC activity measured during hemolytic crisis in SCD patients (60, 67). APC pre-incubation with PPIX resulted in a similar effect concerning the amidolytic activity, which demonstrates the importance of the protoporphyrin ring system for the interaction. Interestingly, ZnPPIX, which is naturally formed in iron deficiency (41), had an even stronger effect on APC. The amidolytic activity was almost completely suppressed. By now, ZnPPIX itself was only reported to be a potent inhibitor of HO-1 reducing APC levels in septic mice (13). However, zinc(II) ions were shown to inhibit the enzymatic activity of APC

towards a peptide substrate and FVa with a binding affinity in the micromolar range (K_D = 7.4 µM) (77). In the present experimental set-up, ZnPPIX seems to have an even higher effect, which again would confirm the significance of the porphyrin ring for interaction. Physiologically, increased levels of ZnPPIX accompanying iron deficiency states were earlier associated with malaria, which in the course of disease development results in hemolysis (27). Under these conditions, ZnPPIX could potentially contribute to a decreased APC amidolytic activity. Our results demonstrate that the inhibitory effect is not only provoked by heme, but also other PPIX derivatives, which may lead to an even stronger effect under physiological conditions.

In further support of our hypotheses, heme and its analogs were capable to inhibit APC's anticoagulant function in an aPTT-based clotting assay. Thereby, heme even enabled the complete elimination of the anticoagulant effect of APC. As one of the classical heme scavenger proteins albumin binds heme and captures it under hemolytic conditions (57, 63). Thus, upon addition of exogenous HSA we could observe that APC is protected from inhibition by heme to a certain extent, fully recovering the anticoagulant function of APC. However, heme applied in the highest possible concentration with respect to the assay conditions, i.e. 100 µM, again induced a reduction of APC's anticoagulant function. It can thus be assumed that massive local increase of heme, as occurring during hemolytic events, leads to an inhibition of the anticoagulant properties of APC (Figure 8). This scenario is conceivable at least in case of a complete saturation of the heme scavengers, since already a comparably low heme concentration of 10 µM could induce the inhibition of APC in our approach.

Furthermore, the heme-APC complex was found to exert a much higher peroxidase-like activity (~512.33%) than heme only (100%), an effect that was also demonstrated earlier for the respective complexes with amyloid beta or fibrinogen (2, 28, 71). Interestingly, we recently reported a peroxidase-like activity of >360% for the complex of heme with the APC-derived peptide WIHGHIRDK (motif **2**) (64). It is therefore highly probable that heme binding to this specific motif is responsible for generating a suitable environment for the binding of H_2O_2 and the oxidation of the substrate. This function might be particularly important during hemolytic events in sepsis, which are associated with the formation of reactive oxygen species (ROS)

such as H_2O_2 (11, 33). Furthermore, heme itself is well-known for its cytotoxic effect through induction of oxidative stress, which, in turn, has been suggested to promote coagulation (18, 42). Again, this would support the procoagulant effect of heme. In addition, APC exerts cytoprotective functions through interaction of its acidic exosite with the receptor PAR-1 (21, 73). Interestingly, the potential heme-binding motif **2** is close to this PAR-1-interaction site in APC (Figure 8C). As a consequence, investigation of the cytoprotective functions of APC in the context of high heme concentrations is of great importance. As reported earlier (62), heme exposure of endothelial cells resulted in a concentration-dependent barrier dysfunction of the endothelial monolayer. Our results obtained using a HUVEC-based barrier assay support these findings. In the presence of APC, however, the cytotoxic effect of heme was partly neutralized indicating that the cytoprotective functions of APC are not inhibited by heme binding. Thus, the interaction between heme and APC seems to be of bilateral nature. While heme partially inhibits the anticoagulant functions of APC, the cytoprotective functions remain intact.

Herein, we report for the first time a direct interaction of heme with the serine protease APC, providing a first evidence for specific binding motifs as well as functional consequences resulting from heme binding to APC. To which extent heme might influence the PC system through interaction with APC depends on time and spatial hierarchy of the simultaneously occurring reactions as well as on concentration of other heme-binding proteins (apart from heme-scavenging proteins) and their respective heme-binding affinity (Figure 7, 8A). However, it is tempting to speculate that heme-induced inactivation of APC contributes to the hypercoagulable state associated with severe hemolysis.

Innovation

Several coagulation factors have been earlier described to be affected by heme, which together with the occurrence of coagulopathies during hemolytic events inevitably suggests a link between hemolysis and coagulation. For the first time we demonstrate transient heme-binding to APC, an anticoagulant serine protease, and identify two potential binding sites. Furthermore, our findings on the functional consequences for APC upon heme binding led us to assume that a heme overload under hemolytic conditions might affect directly the protein C pathway, and consequently exerts an effect on clotting processes.

Materials and Methods

Reagents

Hemin (Fe(III) protoporphyrin IX), in the following designated as heme, was obtained from Frontier Scientific (Logan, Utah, USA). Stock solutions of 1 mM heme were freshly prepared as described earlier (5, 7, 38, 39, 71). The stock solutions were diluted to the desired concentration in the respective assay buffer directly before use. Stock solutions of protoporphyrin IX and zinc protoporphyrin IX (Frontier Scientific, Logan, Utah, USA) were prepared identically to heme. Ferric chloride ($FeCl_3$, anhydrous, Merck, Darmstadt, Germany) was solved 1 mM in water (dd) for stock solution and freshly diluted before use. Plasma-derived human APC (HCAPC-0080, Hematologic Technologies, Essex Junction, Vermont, USA), human α-thrombin (T6884, Sigma-Aldrich, St. Louis, Missouri, USA), recombinant human factor XIIIa (T070; Zedira, Darmstadt, Germany) and human serum albumin (HSA, A3782, Sigma-Aldrich, St. Louis, Missouri, USA) were commercially obtained. The recombinant human APC (Drotrecogin alfa, Xigris®) was obtained from Eli Lilly and Company (Indianapolis, Indiana, USA). As a drug, Xigris® was in use for the treatment of severe sepsis but was withdrawn from the market due to a lack of efficiency. It differs from plasma-derived APC only in its glycosylation pattern, which was demonstrated to result in considerable differences in activity (74, 75). The purity of all proteins was examined by SDS-PAGE.

Surface plasmon resonance (SPR) binding studies

SPR binding studies were performed on a Biacore T200 instrument (GE Healthcare Europe GmbH, Freiburg, Germany) as described earlier (49, 70). APC was dissolved in 10 mM acetate buffer (pH 4.5) in a concentration of 10 µg/ml and immobilized by amine coupling on a CM5 sensor chip (GE Healthcare) to a response unit (RU) of 2261 RU. Binding kinetics were determined by a titration of five consecutive injections with different heme concentrations (187.5 nM, 375 nM, 750 nM, 1500 nM, 3000 nM), prepared by dilution of the stock solution (1 mM) with running buffer. Measurements were performed at 25°C in running buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4) with a flow rate of 30 µl/min in the standard

single-cycle kinetics method (Biacore T200 control software, GE Healthcare). Regeneration of the sensor chip surface was achieved by injection of 10 mM NaOH. Binding kinetics were evaluated by global fitting with the Biaevaluation software (GE Healthcare).

Screening and selection of potential heme-binding sites in APC

As described earlier, potential heme-binding motifs (HBMs) were selected as nonapeptide sequences using the SeqD-HBM search algorithm, which was recently manifested with the webserver HeMoQuest (5, 7, 38, 39, 48, 72). HeMoQuest is a computational approach for the prediction of transient heme binding to proteins (48). After entering the sequence of APC, 10 motifs were shortlisted based on the search criteria~~.~~ (a potential coordinating site (Cys, Tyr or His), a positive net charge and surface-exposed). Five cysteine-based motifs were excluded since the cysteine residues are involved in disulfide bridges (45), and one motif includes a glycosylation site (Asn^{235}) (22), both hindering heme binding. Finally, four motifs were synthesized as nonapeptides for studying their heme-binding properties (see Synthesis, purification and amino acid analysis of APC-derived peptides; Table S1).

Synthesis, purification and amino acid analysis of APC-derived peptides

Automated solid-phase peptide synthesis of APC-derived peptides **1** - **4** was carried out by standard 9-fluorenylmethyl-oxycarbonyl/*tert*-butyl (Fmoc/tBu) strategy on Rink amide MBHA resin, as described before (5, 7, 38, 39, 71). Subsequently, the crude peptides were purified by preparative reverse-phase high-performance liquid chromatography. The purity of the peptides was examined by analytical HPLC (LC-10AT, Shimadzu), the molecular identity was confirmed by electrospray ionization mass spectrometer (ESI-MS, micrOTOF-Q III, Bruker Daltonics GmbH, Bremen, Germany) connected to a Dionex Ultimate 3000 LC 8 (Thermo Fisher Scientific, Dreieich, Germany) (Table S1). As described before (5, 7, 38, 39, 71), amino acid analysis was performed to determine the peptide content, which was taken into consideration for the following studies.

Analysis of heme binding to APC-derived peptides by UV/vis spectroscopy

Heme binding to peptides **1** - **4** was studied by UV/vis spectroscopy as described earlier (39). In brief, different concentrations of heme (0.4 – 40 µM) were incubated for 30 min with the peptide (10 µM) in 100 mM HEPES buffer (pH 7.0). Subsequently, the absorbance spectra (300 – 600 nm) were recorded on a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany). Difference spectra were obtained by subtracting spectra of heme and peptide, respectively, from the spectra of the heme-peptide complexes for each heme concentration. K_D values were determined using GraphPad prism 8.0 software and the equation earlier described (39, 53).

Molecular dynamics and docking simulation studies of the heme-APC complex

First, homology modeling was employed to construct the full-length three-dimensional structure of APC in YASARA structure (version 18.2.7) (34, 35). The program used five initial structures (PDB ID: 1AUT, 3F6U, 2AER, 1W0Y, 3HPT) as templates in the construction of 17 different initial models based on different alignment variants. A hybrid model was built by combining the best parts of the 17 initial models. Finally, the Gla domain of the hybrid model was manually replaced by the Gla domain obtained from an earlier study which contained the calcium ions at their precise locations (51). This model was energy minimized using the steepest descents and stimulated annealing protocol as implemented by the YASARA macro rm_run.mcr. This minimized structure that converged to a total energy of -45552.48 kJ/mol was subjected to a 300 ns molecular dynamics (MD) simulation intended to produce an equilibrated conformational ensemble of the protein. The simulated system attained conformational equilibrium with an RMSD change of less than 2.5 Å after ~150 ns of the simulation and the final 100 ns of the simulation was considered as the production phase from where snapshots were selected for further analysis.

Using selected structures obtained from the production phase of the MD simulation trajectory of the model, we performed focused molecular docking studies of heme to potential HRMs of APC. The snapshots were selected arbitrarily upon visual examination of the trajectory

specifically choosing ones where the coordinating residue of (H or Y) of the potential HRM was found with good surface exposure. The heme molecule was downloaded from the ChemSpider database (ChemSpider ID 16739951) (50). For each potential HRM, a cubic simulation cell (15 Å x 15 Å x 15 Å) was built around the expected coordinating residue. The AutoDock Vina algorithm was used to run docking simulations via YASARA (35, 65). The ensemble docking approach was used by creating a protein receptor ensemble of five high-scoring solutions of the side chain rotamer network at a temperature of 298 K (65). To each rotamer, heme was docked twenty times resulting in a hundred docking poses per run. The resulting poses were clustered to produce a list of ligand-receptor complex conformations, sorted by predicted binding energies and a 5 Å (default value used by Vina) heavy ligand atom RMSD threshold to separate the clusters.

Molecular dynamics (MD) simulations of selected heme-APC docked complexes were conducted on the YASARA version 19.1.27 platform to assess the stability of heme binding to the specific HRMs thereby validating the docking hit that was found (8, 36). Heme docking to APC was considered successful in poses where the distance between the Fe^{3+} ion of heme and the non-protonated nitrogen atom of histidine or the hydroxyl oxygen of tyrosine is ≤ 3 Å. First, selected docked complexes for each HRM were loaded into YASARA as starting structures for the MD simulations. All MD simulations were conducted in the NPT ensemble with a 2 fs time step, at 298 K and a pressure maintained at 1 bar. Periodic boundary conditions were used on the simulation cell which was constructed to have a minimum distance of 10 Å from all protein atoms. Long range forces were computed with a cutoff of 8 Å and the particle-mesh Ewald method was used (12). Three rounds of simulations were conducted for each complex, the first of which was run for 20 ns. Furthermore, two independent validation runs for 50 ns each were conducted by creating mildly altered initial configurations of the system via energy minimizations (Figure S7). The first 20 ns run was used to verify if the docked complex was stable in the first place and the subsequent two 50 ns validation runs were done to be sure that the binding persists even when the initial coordinates of the complex are altered due to the fact that MD simulations are highly sensitive to the initial configuration of the system (4).

Analyses of the MD trajectories were done both in YASARA as well as in VMD (version 1.9.3) by first converting the trajectory into the GROMACS XTC format (26, 30). Molecular graphics were created from UCSF Chimera (1.13.1) (52), and plots were generated using GraphPad prism 8.0 software. To enable a qualitative comparison of the heme-protein interaction between various motifs, the binding energy computed by the Poisson-Boltzmann method as implemented by the md_analyzebindingenergy.mcr macro in YASARA was used on the trajectories of the 50 ns validation runs (15). The energies computed here are not to be considered in any sense comparable to the real or experimentally obtained binding potentials, but only as arbitrary measures for relative comparison to quantify the interaction of heme to the different motifs.

Amidolytic activity of human APC, thrombin and factor XIIIa (FXIIIa) in the presence of heme

The impact of heme on the catalytic activity of APC was spectrophotometrically analyzed on a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany) based on a diagnostic assay, which is commonly used for the quantification of functionally active protein C (Berichrom® Protein C). APC (100 nM) was pre-incubated with different concentrations of heme (0 – 50 µM) for 15 minutes at room temperature in the dark. 90 µl of the heme-APC complex were placed into a 96-well microplate (Greiner Bio-One GmbH, Frickenhausen, Germany) and 10 µl of the chromogenic substrate p-Glu-Pro-Arg-MNA (Berichrom® Protein C kit, Siemens Healthcare Diagnostics, Erlangen, Germany) was added in a concentration varying from 0 – 2.5 mM. The assay was performed in HEPES buffer (100 mM, pH 7.4). K_M was obtained by non-linear regression fitting using the Michaelis-Menten equation, whereas the inhibition constant K_i was graphically analyzed using Dixon plot (GraphPad prism 8.0 software). Performing the assay with a constant substrate concentration of 300 µM allowed for the comparison of the effect of heme with that of other porphyrin analogs (zinc protoporphyrin IX and protoporphyrin IX) and iron(III) chloride. Furthermore, the impact of heme on recombinant APC (rAPC, Xigris®) was investigated. The concentration of rAPC

needed to be adapted to 50 nM. A range of heme concentrations (0 – 10 µM) was applied. The same procedure was used for thrombin, but with a final enzyme concentration of 25 nM and heme in concentrations of 0 – 50 µM. Again, p-Glu-Pro-Arg-MNA served as the substrate. The activity of FXIIIa was monitored in Tris buffer (0.05 M Tris, 0.154 M NaCl, 0.01 M $CaCl_2$, pH 7.5) using H-Tyr-Glu(pNA)-Val-Lys-Val-Ile-Gly-NH_2 (100 µM) as the substrate (25). The final concentration of FXIIIa was 400 nM. Heme was added in final concentrations of 0 – 50 µM. The increase of absorbance due to substrate conversion was monitored every 10 s for 20 min at 405 nm. The remaining activity of the enzymes as the change of absorbance per second was normalized against the basal activity without any potential ligand. Data was analyzed as the mean of triplicate, and the IC_{50} value was determined by fitting the curves with non-linear regression (GraphPad prism 8.0 software).

Monitoring the peroxidase-like activity of the heme-APC complex

As described earlier (64, 71), 42 µM of heme was pre-incubated with 42 µM of pdAPC for 30 min in PBS buffer (pH 7.4). A mixture of TMB (1.66 mM) and H_2O_2 (163.2 mM) served as the substrate. After transfer of 10 µl of the heme-APC complex (final concentration 1 µM) to 200 µl of the substrate, the absorbance was detected at 652 nm. For comparison, the same experiment was performed with heme as well as APC only as controls. Data was normalized against the activity of heme and evaluated as the mean of triplicates.

Anticoagulant activity of APC in the presence of heme

The impact of heme on the anticoagulant activity of pdAPC was analyzed by using an activated partial thromboplastin time (aPTT)-based clotting assay, performed with the semi-automatic ball coagulometer KC10 (Amelung, Lemgo, Germany) (24). APC (5 nM) was incubated with different concentrations of heme (0-100 µM) for 1 hour in DPBS buffer (with Ca^{2+} and Mg^{2+}) or in DPBS with 0.1% HSA (with Ca^{2+} and Mg^{2+}). After addition to the 10-fold volume of citrated pool plasma (in-house preparation), 100 µl of the respective mixture was immediately incubated with 100 µl of the contact phase activator Actin FS (Siemens Healthcare

Diagnostics, Erlangen, Germany) at 37°C for 3 min followed by the addition of 100 µl of 30 mM $CaCl_2$ solution (Siemens Healthcare Diagnostics, Erlangen, Germany), as described before (24). As a control, pool plasma containing the same amount of heme without addition of exogenous APC was subjected to the assay system. Clotting times, given in seconds, were automatically determined by the coagulometer. Data were evaluated as the mean of triplicates (GraphPad prism 8.0 software). In a second approach, the impact of heme with the lowest potent concentration was compared to that of its analogs ZnPPIX and PPIX (10 µM in the absence and 100 µM in the presence of 0.1% HSA).

Evaluation of the cytoprotective function of APC in the presence of heme

The endothelial barrier permeability assay (Merck) was used to determine the cytoprotective function of pdAPC after preincubation with heme. As described before (58), HUVECs were seeded (2.5 x 10^4 cells/well) onto collagen-coated culture inserts of a 96-well plate and subsequently cultured in endothelial cell growth medium (ECGM, C-22010, PromoCell, Heidelberg, Germany) for four days to reach maximum confluency. The upper chamber contained 125 µl and the lower chamber 250 µl of ECGM. One hour before starting the experiment, ECGM was exchanged with phenol red-free endothelial cell basal medium (ECBM, C-22215, PromoCell, Heidelberg, Germany). APC (20 nM) was preincubated with heme (120 µM) for 30 min in phenol red-free ECBM. Subsequently, the mixture was applied to the upper chamber and the cells were incubated for 3 hours at 37°C, 5% CO_2. As controls, buffer, APC and heme were separately incubated with the cell monolayer. After washing the cells three times with PBS, the APC/heme mixture was replaced by either thrombin (20 nM) or ECBM as a control for 30 min at 37°C. In the following, the content of the upper and lower chamber was replaced by fluorescein isothiocyanate (FITC)-labelled dextran (75 µl of 1:40 dilution in phenol red-free ECBM) and 250 µl phenol red-free ECBM, respectively, and incubated for 20 min at room temperature. Finally, the determination of the fluorescence intensity (λ_{ex} = 485 nm, λ_{em} = 535 nm) with a Synergy 2 microplate reader allowed for the

quantitation of the flux through the endothelial cell monolayer at a rate proportional to the permeability. Data are presented as the mean of quadruplicates.

Statistical analysis

Ordinary one-way ANOVA with post-hoc Tukey's multiple comparisons test was used to calculate the indicated p-values. A p-value of 0.05 was assumed to be significant. Calculations were performed by using GraphPad prism 8.0 software.

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Authorship contributions

MTH, NSH, BP, and DI conceived and designed the study. MTH performed the experiments and collected the data. NSH performed the barrier protection assay. NA, AAPG, and AB carried out the computational studies. MTH, NSH, BP, AAPG, AB, and DI analyzed the data. BP and JO contributed analysis tools. The manuscript was written through contributions of all authors.

Author disclosure statement: The authors declare no competing financial interests.

List of abbreviations

2D-NMR	=	two-dimensional nuclear magnetic resonance spectroscopy
APC	=	activated protein C
aPTT	=	activated partial thromboplastin time
ANOVA	=	Analysis of variance
cwEPR	=	continuous-wave electron spin resonance spectroscopy
ECGM	=	endothelial cell growth medium
ECGM	=	endothelial cell basal medium
EGF	=	epidermal growth factor
EPCR	=	endothelial protein C receptor
FII	=	prothrombin
FIIa	=	thrombin
FITC	=	fluorescein isothiocyanate
FIX	=	factor IX
FIXa	=	activated factor IX
FMx	=	crosslinked fibrin
FV	=	factor V
FVa	=	activated factor V
FVII	=	factor VII
FVIIa	=	activated factor VII
FVIII	=	factor VIII
FVIIIa	=	activated factor VIII
FX	=	factor X
FXa	=	activated factor X
FXI	=	factor FXI
FXIa	=	activated factor FXI
FXII	=	factor XII
FXIIa	=	activated factor XII

FXIII	=	factor XIII
FXIIIa	=	activated factor XIII
H_2O_2	=	hydrogen peroxide
HBM	=	heme-binding motif
HEPES	=	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HO-1	=	heme oxygenase 1
HRM	=	heme-regulatory motif
HUVECs	=	human umbilical vein endothelial cells
IC_{50}	=	half maximal inhibitory concentration
K_a	=	association rate constant
K_d	=	dissociation rate constant
K_D	=	equilibrium dissociation constant
K_i	=	inhibitor constant
K_M	=	Michaelis-Menten constant
MD	=	molecular dynamics
ns	=	not statistically significant
PAR1	=	protease-activated receptor 1
PC	=	protein C
PCI	=	protein C inhibitor
pdAPC	=	plasma-derived activated protein C
PPIX	=	protoporphyrin IX
PS	=	protein S
rAPC	=	recombinant activated protein C (Drotrecogin alfa, Xigris®)
ROS	=	reactive oxygen species
rRaman	=	resonance Raman spectroscopy
RU	=	response unit
SCD	=	sickle cell disease
SeqD-HBM	=	sequence-based detection of heme binding motifs

S1P	=	sphingosine-1-phosphate
TF	=	tissue factor
TLR4	=	Toll-like receptor 4
TM	=	thrombomodulin
TMB	=	3,3',5,5'-tetramethylbenzidine
Tris	=	2-Amino-2-(hydroxymethyl)propane-1,3-diol
ZnPPIX	=	zinc protoporphyrin IX

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Figures

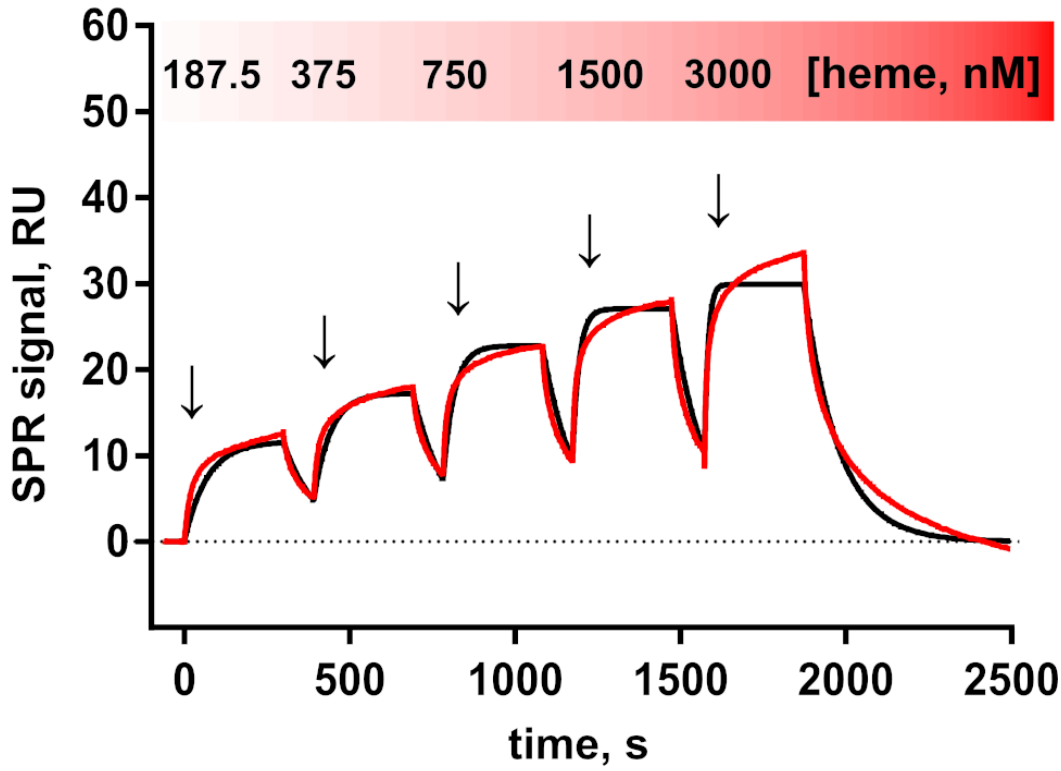

Figure 1. Heme binds to APC. Single cycle kinetic SPR analysis of the heme interaction (187.5 - 3000 nM) with APC revealed at least one specific heme-binding site with a binding affinity in the nanomolar range (K_D ~ 400 ± 47 nM). The K_D value was calculated by using a kinetic global fit (red curve). Moreover, the determined stoichiometry of 1:1.3 indicates that there are overlaying binding events of specific and/or unspecific interactions. The fast association (k_a ~2.06 x 104 s^{-1}) and dissociation rates (k_d ~8.08 x 10^{-3} s^{-1}) refer to a transient interaction of heme and APC.

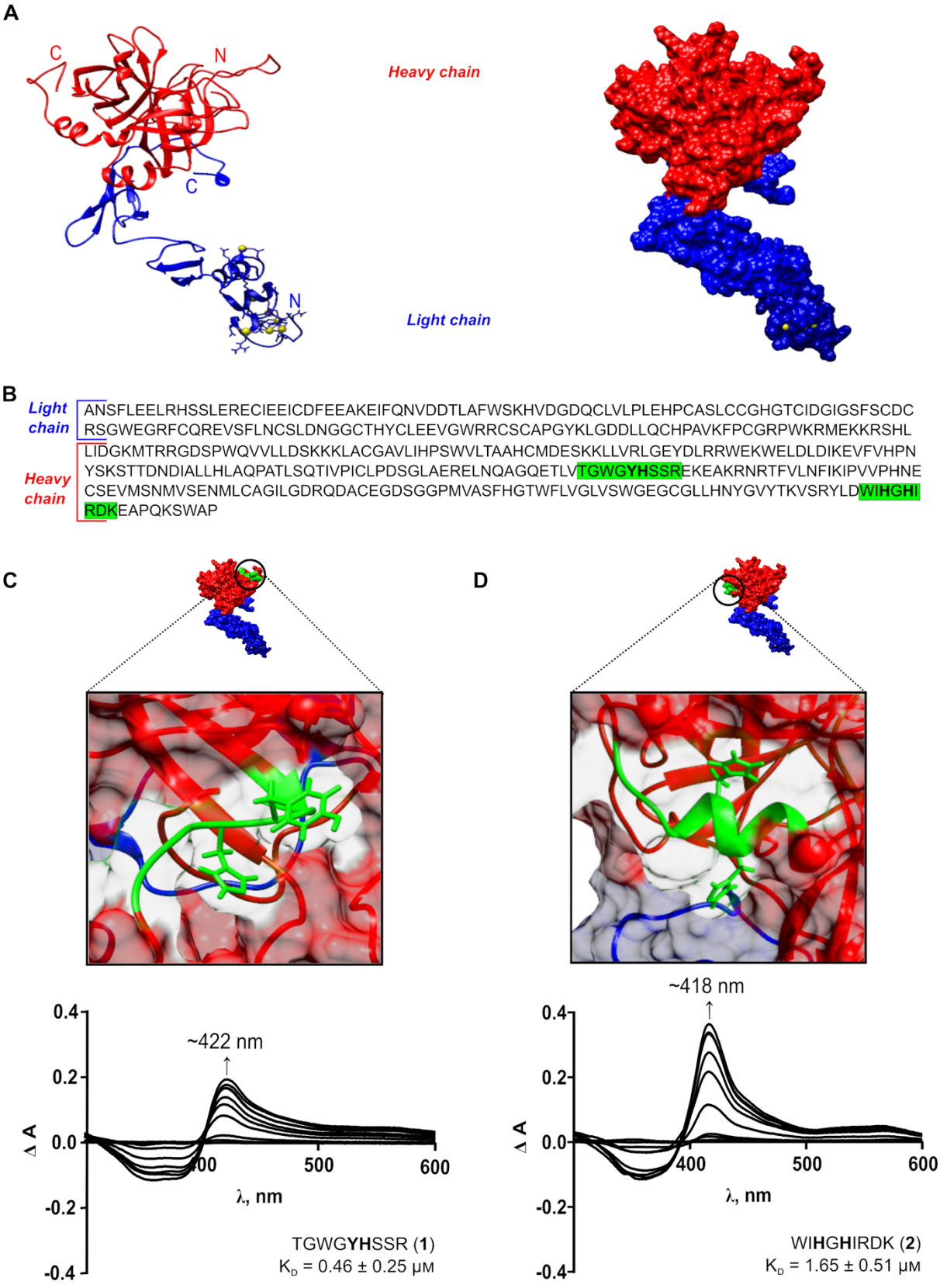

Figure 2. Identification of heme-regulatory motifs (HRMs) on the surface of APC. (A) Homology model of APC depicted as a ribbon structure (left) and a space-filling model (right), with the heavy chain in red, and the light chain in blue with calcium ions (yellow). **(B)** Sequence

of APC divided into light and heavy chain. HRMs are highlighted in green. **(C, D)** Motifs **1** (TGWGYHSSR) and **2** (WIHGHIRDK) are found within the heavy chain, here shown as a zoom-in into the whole structure of APC. The potential coordination sites are accentuated by stick bonds (above). The affinity for heme binding to these stretches was estimated by UV/vis binding studies of heme to motif-derived nonapeptides. These peptides (each 10 μM) were incubated with heme (0 – 40 μM). Depicted are the difference spectra. For both peptides a high affinity for heme was demonstrated (below).

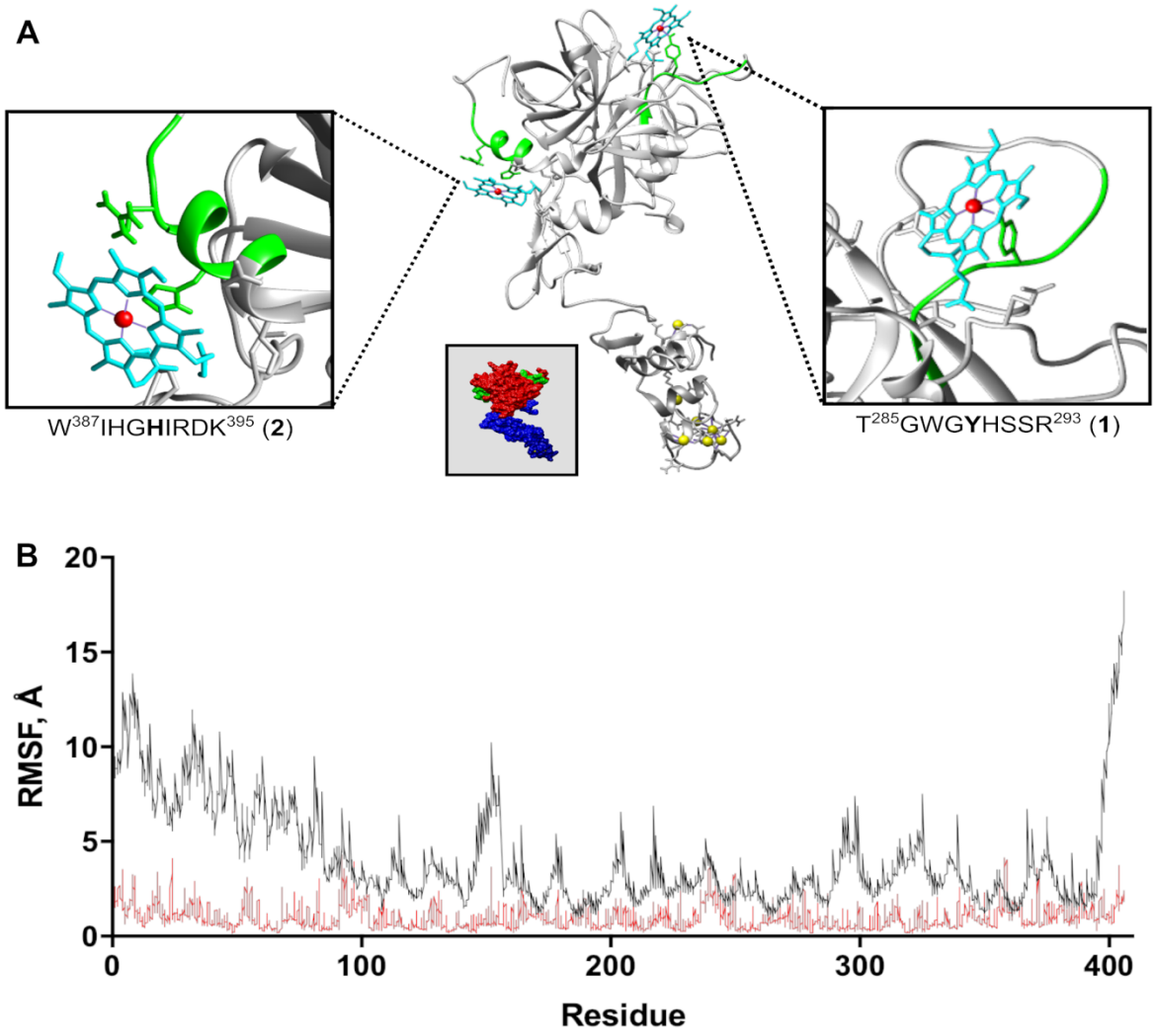

Figure 3. Heme coordination by APC via Tyr^{289} and His^{391} has a structural impact on APC. (A) Two heme molecules docked to APC through interaction with Tyr^{289} (right) and His^{391} (left). The respective motifs are highlighted in green within the structure. **(B)** The per residue root mean square fluctuation (RMSF) plots were computed from two different 300 ns long MD simulations run on APC. The RMSF plot from the simulation with no heme molecules bound to APC (black line) has a flexibility characteristic of most proteins with the terminal ends of the protein showing higher mobility. In comparison, in case of APC with heme molecules bound to both motifs **1** and **2** by molecular docking, the entire protein has a pronounced loss of mobility as indicated by the RMSF plot (red line).

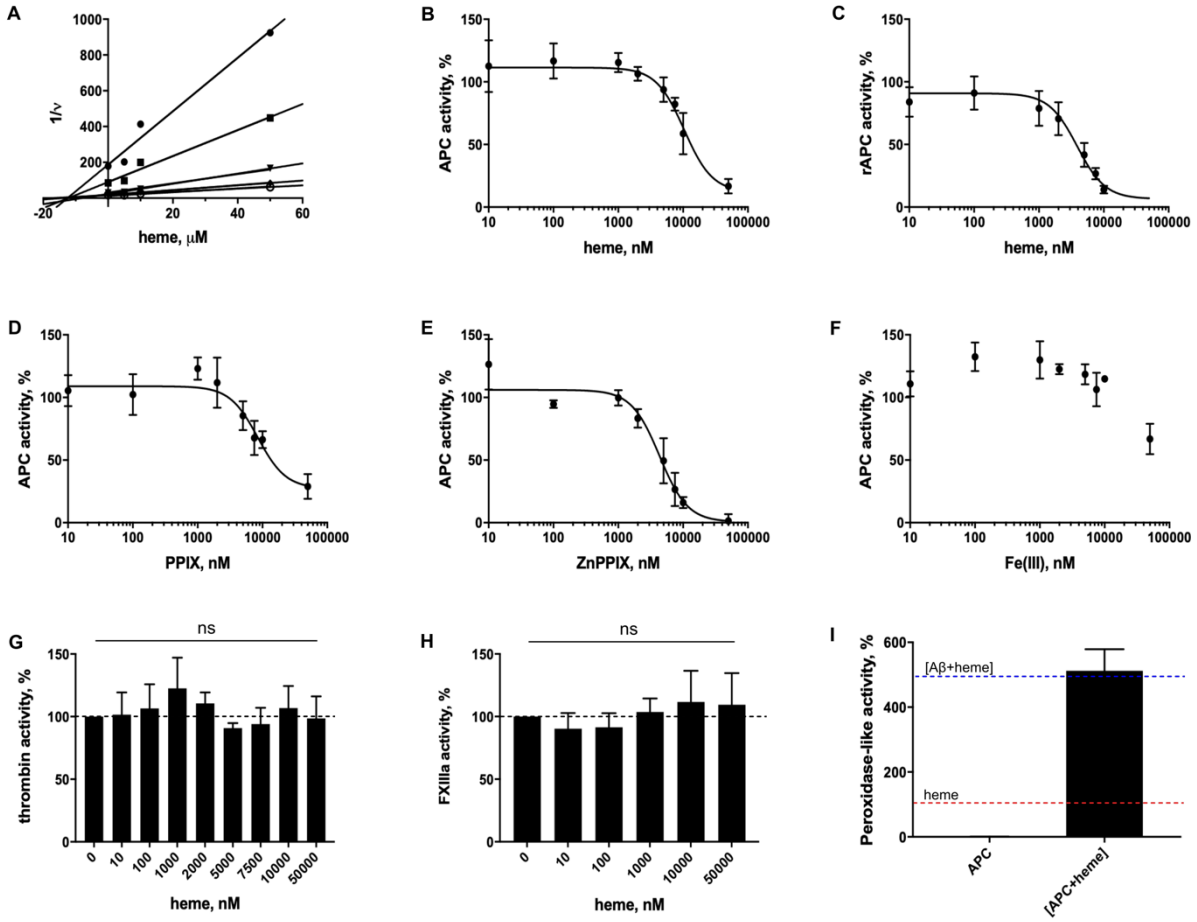

Figure 4. Effect of heme on the amidolytic activity of APC. (A) Evaluation of K_i (12.56 ± 2.31 µM) of heme (0 – 50 µM) for pdAPC (100 nM) with different substrate concentrations (● 0.05 mM, ■ 0.1 mM, ▼ 0.5 mM, ♦ 1 mM, ○ 2.5 mM) using Dixon plot (reciprocal reaction rate ν plotted against increasing concentrations of heme as the inhibitor). Inhibition of the amidolytic activity of **(B)** pdAPC (100 nM), and **(C)** rAPC (50 nM) by heme (0 - 50 µM). IC_{50} values were determined to be 10.41 ± 1.24 µM and 3.88 ± 0.87 µM, respectively. **(D, E)** Inhibition of pdAPC by heme derivatives PPIX and ZnPPIX with IC_{50} values of 8.46 ± 1.68 µM and 4.33 ± 0.87 µM. **(F)** The effect of $FeCl_3$ on pdAPC in the same concentration range seems to be negligible due to the fact that only the highest concentration shows a minimal effect reducing the activity to ~66.83%. **(G, H)** In comparison to APC, no statistically significant (ns) effect of heme was observed on the enzymatic activity of α-thrombin **(G)** and rFXIIIa **(H)**, as analyzed by applying the one-way ANOVA test. The black dashed lines represent the normalized activity of the enzymes without addition of heme (100%), respectively. Values are normalized to the

enzymatic activity of the respective enzymes renouncing the addition of porphyrins or iron ions. **(I)** Peroxidase-like activity of heme is increased in complex with APC ([APC+heme]) up to 512.33 ± 66.50%. The dashed line represents the normalized activity of heme (100%; red) and the heme-amyloid beta complex (~500% (71); [Aβ+heme], blue) for comparison.

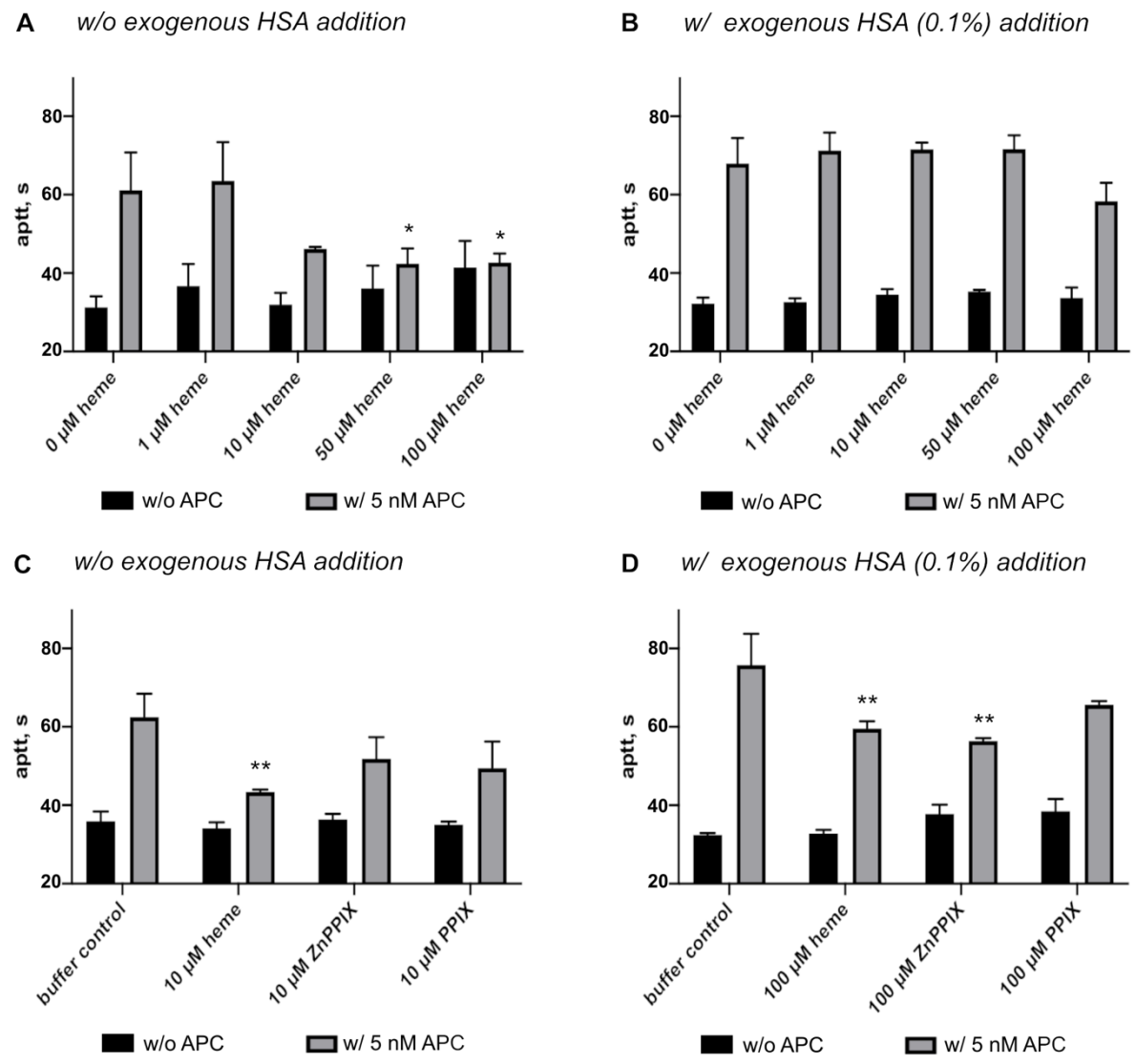

Figure 5. Heme-mediated inhibition of the anticoagulant function of APC. (A, B) Dose-dependent inhibition of pdAPC by heme, observed in a conventional aPTT-based clotting assay. Preincubated with APC (5 nM), the lowest effective heme concentration was 10 µM (without additional HSA, **(A)**) and 100 µM (in the presence of 0.1% HSA, **(B)**), respectively. **(C, D)** Furthermore, the heme analogs ZnPPIX and PPIX were also capable to inhibit the anticoagulant function of APC in both settings. Statistically significant differences in comparison to the sample without heme are shown according to one-way ANOVA. *, $p \leq 0.05$; **, $p < 0.01$.

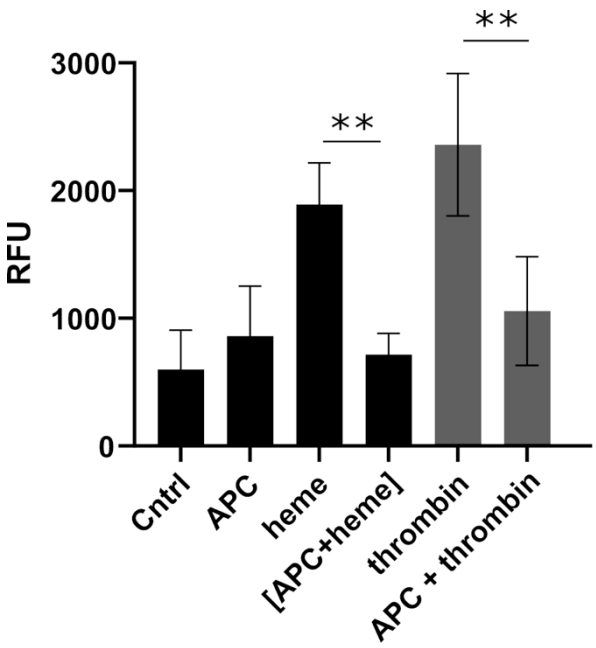

Figure 6. APC's barrier protective function is not altered by heme. Heme exposure (120 μM) causes an increase of the endothelial cell monolayer permeability, which is completely prevented by 30 min preincubation of heme with APC (20 nM). While thrombin (20 nM) alone significantly increases the permeability of the cell monolayer, APC (10 nM) protects from thrombin-induced endothelial barrier permeability, which is determined here as fluorescence intensity as a measure for the flux of FITC-labeled dextran through the endothelial cell monolayer. RFU, relative fluorescence units; **, $p < 0.01$.

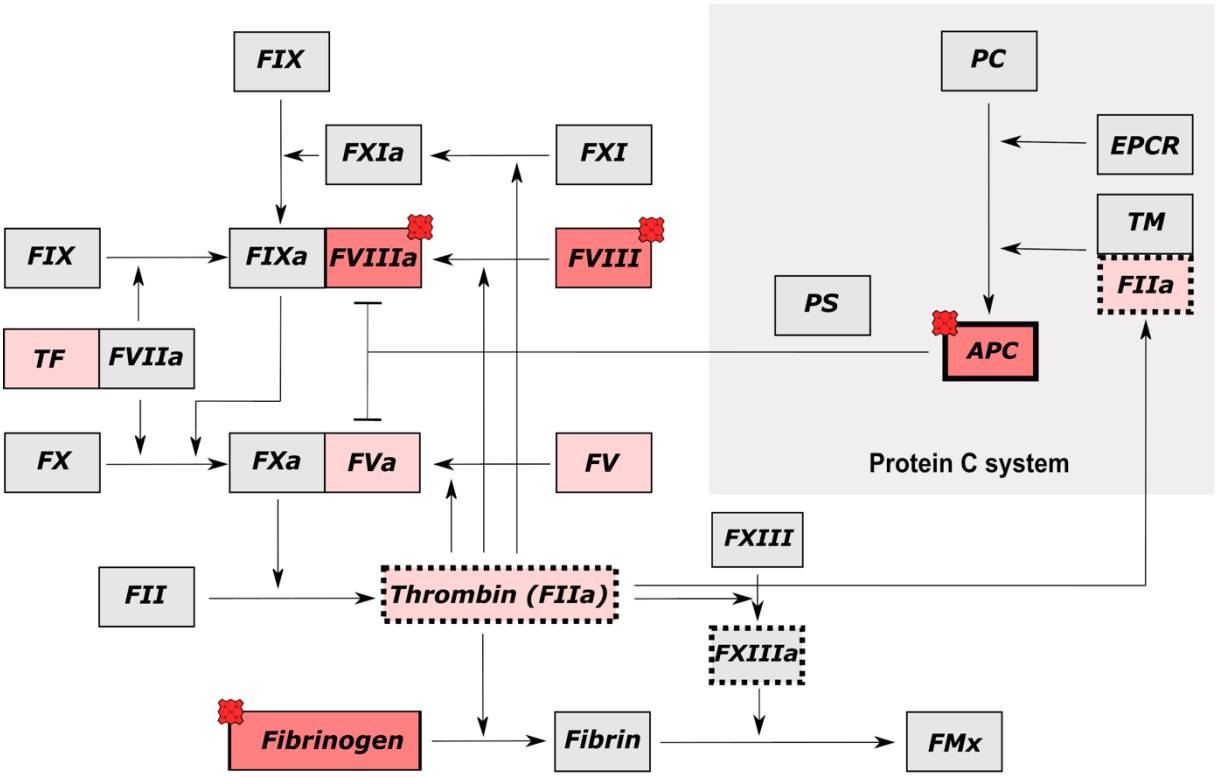

Figure 7. APC is the first clotting inhibitor that is reported to bind heme. Previously, there was no report on APC as a heme-binding protein. In this study, binding of heme to APC was demonstrated with a K_D value in the nanomolar range. This resulted in a change of its amidolytic activity, whereas thrombin (FIIa) and FXIIIa were not affected in their activity in the presence of heme (dashed boxes). An impact of heme or hematin on the plasma levels or function of other coagulation factors was earlier reported (light red boxes) (19, 31, 57). FVIII(a) and fibrinogen are known to bind heme (dark red boxes, accentuated by the heme symbol) (31, 55). For other factors there is no link to heme binding reported so far (grey boxes).

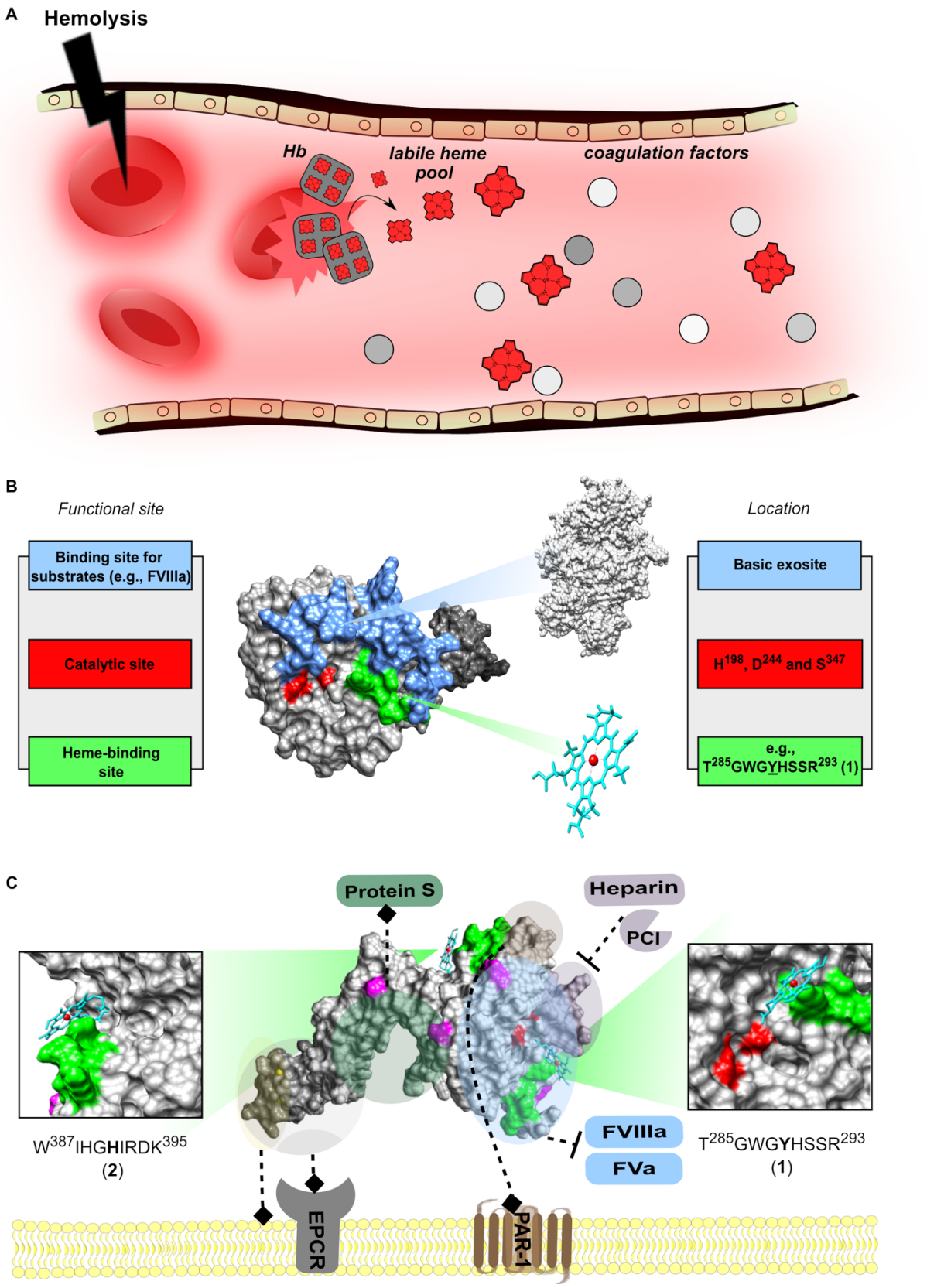

Figure 8. Hemolysis-derived labile heme and its interaction with APC. (A) Under hemolytic conditions, the level of labile heme increases as a consequence of RBC rupture and subsequent degradation of hemoglobin (Hb) (57). This leads to simultaneous intravascular

occurrence of labile heme and clotting factors. Consequently, heme can exert its regulatory function by affecting different coagulation factors (different gray circles) with respect to their plasma levels and/or their functional activity (19, 31, 57). **(B)** Regulatory heme binding to proteins occurs to short surface-exposed sequence stretches. In case of APC, the heme-binding site (green) is close to its catalytic site (red). Therefore, heme-binding to APC occurs not through a direct binding to the catalytic site. There is thus no direct correlation between the heme-binding affinity of APC and the inhibitory effect of heme on APC. In contrast, heme binding, especially if involved in specific binding sites (e.g., FVa- and FVIIIa (PDB: 2r7e)-binding sites; blue), can induce conformational changes that might explain the effect on the activity and/or function of APC. **(C)** Mapping the interaction sites of APC with its interaction partners in comparison with the sites of the potential heme-binding motifs (green) reveal that motif **1** is very close to the catalytic triad (red) of APC with high proximity to its active site serine residue (zoom-in, right). In addition, it is part of the important sites for interaction with the clotting factors FVa and FVIIIa (blue), which serves as an explanation for the inhibitory effect of heme on APC's anticoagulant function. Motif **2** (zoom-in, left) is closer to the interaction site of APC with PAR-1 (brown), suggesting a potential relevance of heme for the cytoprotective function of APC. Furthermore, interaction sites for phospholipids (yellow), EPCR (grey), Protein C inhibitor (PCI), and the glycosaminoglycan heparin (purple) are labelled (69). Interaction sites and respective interaction partners have the same color and are connected by dashed lines. Glycosylation sites are highlighted (magenta) (22).

4.2.2 Summary

Surface plasmon resonance spectroscopic binding analysis initially confirmed heme binding to APC with a binding affinity in the nanomolar range (K_D ~400 nM) and both rapid association and dissociation of heme, supporting the assumption of a transient nature of this interaction. In addition, a stoichiometry of 1:1.3 (APC:heme) suggested the presence of one to two binding sites for heme. Consequently, screening of the primary sequence and structure of APC as well as the subsequent analysis on the peptide level revealed two highly favorable HRMs, a YH- and a HXH-based motif. Both motifs are located within the serine protease domain of the enzyme at the site of interaction with its natural substrates FVa and FVIIIa. The YH-motif is very close to the catalytic triad, suggesting a possible impact of heme binding on the enzymatic activity of APC. Subsequent analyses of the function of APC in the presence of heme revealed that heme was capable of inhibiting the amidolytic activity of plasma-derived APC (K_i ~12.56 μM, IC_{50} ~10.41 μM) as probed by a colorimetric assay with a peptidic substrate, thereby providing a potential explanation for the decreased activity of APC under hemolytic conditions. In contrast, the enzymatic activity of FXIIIa and thrombin was not affected by heme. In complex with heme, the peroxidase-like activity of heme was significantly increased, which could tend towards a procoagulant effect *in vivo*. Most importantly, heme abolished the anticoagulant function of APC in a concentration-dependent manner in a plasma-based clotting assay, up to a complete inhibition, which would support procoagulant reactions under hemolytic conditions. However, the cytoprotective function of APC was not affected, on the contrary, APC even protects endothelial cells from heme-induced apoptotic signaling in a barrier-protection assay with cultured human umbilical vein endothelial cells. Thus, the interaction between APC and heme seems to be as versatile as both involved parties and extends the knowledge of heme-regulated proteins within the blood coagulation system.

4.3 A computational approach for mapping heme biology in the context of hemolytic disorders

Original research article

Authors*: Farah Humayun, Daniel Domingo-Fernández, Ajay Abisheck Paul George, Marie-Therese Hopp, Benjamin F. Syllwasschy, Milena S. Detzel, Charles Tapley Hoyt, Martin Hofmann-Apitius, and Diana Imhof

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*__Contribution:__ D.I., M.H.-A., and D.D.-F. conceived and designed the study. F.H. curated the data and conducted the main analysis supervised by A.A.P.G., D.I., and D.D.-F. M.-T.H., B.F.S., M.S.D., and A.A.P.G. assisted in selecting the corpora and interpreting the results. C.T.H. designed the curation guidelines and implemented the Python package. D.D.-F., F.H., C.T.H., M.-T.H., B.F.S., M.S.D., and D.I. wrote and reviewed the manuscript.

4.3.1 Introduction

Researchers made great progress in the characterization of heme's various functions in hemolytic diseases, in particular with regard to its proinflammatory role.[6–11] As already suggested for part of the procoagulant functions of heme, activation of the receptor TLR4 has been described as the mechanism for proinflammatory signaling of heme.[221,226,228] However, the knowledge on influenced effector molecules is very limited and, if known, not interconnected. Underlying signaling pathways are therefore largely unknown. In the past, for other pathologies, such as cancer or neurodegenerative diseases, knowledge-driven disease modeling approaches have been proven to be successful in analyzing and networking available data and thus, supporting and promoting the understanding of underlying biological processes.[454–456] Within the following publication, data from 46 heme-related articles is manually extracted and curated by using the biological expression language (BEL).[457] Subsequent pathway enrichment and crosstalk analysis allows for the detailed investigation of the network.

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A Computational Approach for Mapping Heme Biology in the Context of Hemolytic Disorders

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Heme is an iron ion-containing molecule found within hemoproteins such as hemoglobin and cytochromes that participates in diverse biological processes. Although excessive heme has been implicated in several diseases including malaria, sepsis, ischemia-reperfusion, and disseminated intravascular coagulation, little is known about its regulatory and signaling functions. Furthermore, the limited understanding of heme's role in regulatory and signaling functions is in part due to the lack of curated pathway resources for heme cell biology. Here, we present two resources aimed to exploit this unexplored information to model heme biology. The first resource is a terminology covering heme-specific terms not yet included in standard controlled vocabularies. Using this terminology, we curated and modeled the second resource, a mechanistic knowledge graph representing the heme's interactome based on a corpus of 46 scientific articles. Finally, we demonstrated the utility of these resources by investigating the role of heme in the Toll-like receptor signaling pathway. Our analysis proposed a series of crosstalk events that could explain the role of heme in activating the TLR4 signaling pathway. In summary, the presented work opens the door to the scientific community for exploring the published knowledge on heme biology.

Keywords: heme, hemolytic disorders, signaling pathways, knowledge graphs, biological expression language

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INTRODUCTION

Heme is an iron ion-coordinating porphyrin derivative essential to aerobic organisms (Zhang, 2011). It plays a crucial role as a prosthetic group in hemoproteins involved in several biological processes such as electron transport, oxygen transfer, and catalysis (Smith and Warren, 2009; Zhang, 2011; Kühl and Imhof, 2014; Poulos, 2014). Besides its indispensable role in hemoproteins, it can act as a damage-associated molecular pattern leading to oxidative injury, inflammation, and consequently, organ dysfunction (Jeney, 2002; Wagener et al., 2003; Dutra and Bozza, 2014). Plasma scavengers such as haptoglobin and hemopexin bind hemoglobin and heme, respectively, thus keeping the concentration of labile heme at low concentrations (Smith and McCulloh, 2015). However, at high concentrations of hemoglobin and, consequently heme, these scavenging proteins get saturated, resulting in the accumulation of biologically available heme (Soares and Bozza, 2016). With respect to hemolytic diseases, the formation of labile heme at harmful concentrations has been a subject of research for some years now (Roumenina et al., 2016; Soares and Bozza, 2016; Gouveia et al., 2017).

Biomedical literature is an immense source of heterogeneous data that are dispersed throughout hundreds of journals. Furthermore, the majority of the results are scattered and published as unstructured free-text, or at best, presented in tables and cartoons representing the experimental study or biological processes and pathways. These shortcomings, combined with the exponential growth of biomedical literature, prevent the healthcare community and individual researchers from being aware of all the available information and knowledge in the literature. With the introduction of new technologies and experimental techniques, researchers have made significant advances in heme-related research and its role in the pathogenesis of numerous hemolytic diseases such as sepsis (Larsen et al., 2010; Effenberger-Neidnicht and Hartmann, 2018), malaria (Ferreira et al., 2008; Dey et al., 2012), and β-thalassemia (Vinchi et al., 2013; Conran, 2014; Garcia-Santos et al., 2017). In these diseases, large amounts of heme are released from ruptured erythrocytes and can potentially wreak havoc (Tolosano et al., 2010). Thus, it is crucial to develop new strategies that capture and exploit the vast amount of literature knowledge surrounding heme to better understand its mechanistic role in hemolytic disorders.

Biological knowledge formalized as a network can be used by clinicians as research and information retrieval tools, by biologists to propose *in vitro* and *in vivo* experiments, and by bioinformaticians to analyze high throughput *-omics* experiments (Catlett et al., 2013; Ali et al., 2019). Further, they can be readily semantically integrated with databases and other systems biology resources to improve their ability to accomplish each of these tasks (Hoyt et al., 2018). However, enabling this semantic integration requires organizing and formalizing the knowledge using specific vocabularies and ontologies. Although this endeavor involves significant curation efforts, it is key to the success of the subsequent modeling steps. Therefore, in practice, knowledge-based disease modeling approaches have been conducted only for major disorders such as cancer (Kuperstein et al., 2015) or neurodegenerative disorders (Mizuno et al., 2012; Fujita et al., 2014). In summary, while the scarcity of mechanistic information and the necessary amount of curation often impede launching the aforementioned approaches, modeling and mining literature knowledge provide a holistic picture of the field of interest. Furthermore, the underlying models derived from such approaches have a broad range of applications including hypothesis generation, predictive modeling and drug discovery.

Here, we present two resources aimed at assembling mechanistic knowledge surrounding the metabolism, biological functions, and pathology of heme in the context of selected hemolytic disorders. The first resource is a terminology formalizing heme-specific terms that have until now not been covered by other standard controlled vocabularies. The second resource is a heme knowledge graph (HemeKG), that is, a network comprising more than 700 nodes and more than 3,000 interactions. It was generated from 46 selected articles as the first attempt of modeling the knowledge, which is available from more than 20,000 heme-related publications. Finally, we demonstrate both resources by analyzing the crosstalk between heme biology and the TLR4 signaling pathway. The results of this analysis suggest that the activation profile for labile heme as an extracellular signaling molecule through TLR4 induces cytokine and chemokine production. However, the underlying molecular mechanism and individual pathway effectors are not fully understood and need further exploration.

MATERIALS AND METHODS

This section describes the methodology used to generate the mechanistic knowledge graph and its supporting terminology. Subsequently, it outlines the approach followed to conduct the pathway crosstalk analysis. A schematic diagram of the methodology is presented in **Figure 1**.

Knowledge Modeling

In order to identify recently published articles (i.e., published in the last 10 years) describing the role of heme in hemolytic disorders, PubMed was queried with the following: ("heme" AND "hemolysis") OR ("heme" AND "thrombosis") OR ("heme" AND "inflammation") AND ("2009"[Date – Publication]: "3000"[Date – Publication]). The resulting 3,108 articles were manually filtered by removing articles that were deemed too general or lacked a biochemical focus, as judged by expert opinion. After this filtering step, 6 reviews and 40 original research articles were selected for knowledge extraction and modeling. Knowledge was manually extracted and curated from this selected corpus using the official Biological Expression Language (BEL) curation guidelines from http://openbel.org/language/version_2.0/bel_specification_version_2.0.html and http://language.bel.bio as well as additional guidelines from https://github.com/pharmacome/curation.

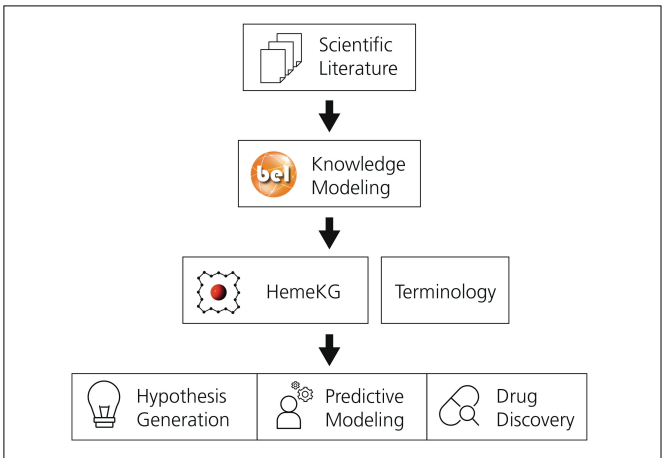

FIGURE 1 | The workflow used to generate the supporting terminology and HemeKG. The first step involves the selection of relevant scientific literature. Next, evidence from this selected corpus is extracted and translated into BEL to generate a computable knowledge assembly model, HemeKG. In parallel to the modeling task, a terminology to support knowledge extraction of articles about the heme molecule was built. Finally, HemeKG can be used for numerous tasks such as hypothesis generation, predictive modeling and drug discovery.

Evidence from the selected corpus was manually translated into BEL statements together with their contextual information (e.g., cell type, tissue and dosage information). For instance, the evidence "Heme/iron-mediated oxidative modification of LDL can cause endothelial cytotoxicity and – at sublethal doses – the expression of stress-response genes" (Nagy et al., 2010) corresponds to the following BEL statement:

SET Cell = "endothelial cell"
a(CHEBI:"oxidised LDL") pos bp(MESH:"Cytotoxicity, Immunologic").

Generation of a Supporting Terminology

During curation, a terminology was generated to support the standardization of domain-specific terminology encountered during the curation of articles related to the heme molecule. The aim of the terminology is to catalog and harmonize terms not present in other controlled vocabularies such as ChEBI (Degtyarenko et al., 2007) for chemicals, or Gene Ontology [GO; (Ashburner et al., 2000)] and Medical Subject Headings [MeSH; (Rogers, 1963)] for pathologies. Thus, each term was checked by two experts in the field assisted by the Ontology Lookup Service [OLS; (Cote et al., 2010)] to avoid duplicates with other terminologies or ontologies. Furthermore, we required that each entry included the following metadata: an identifier, a label, a definition, an example of usage in a sentence, and references to articles in which it was described. Furthermore, a list of synonyms was also curated in a separate file to facilitate the use of the terminology in annotation or text mining tasks. The supporting terminology is included in the **Supplementary Material** and can also be found at https://github.com/hemekg/terminology.

Analyzing Pathway Crosstalk Between Heme and the Toll-Like Receptor Signaling Pathway

Crosstalk analysis aims to study how two or more pathways communicate or influence each other. While there exist, numerous methodologies designed to investigate pathway crosstalk, the majority of these approaches exclusively quantify such crosstalk based on the overlap between a pair of pathways without delving into the nature of the crosstalk (Donato et al., 2013). In this section, we demonstrate how combining knowledge from HemeKG with a canonical pathway reveals mechanistic insights on the crosstalk between two different pathways.

Because of the amount of effort required to manually analyze crosstalk across multiple pathways, we conducted a pathway enrichment analysis on three pathway databases [i.e., KEGG Kanehisa et al., 2016), Reactome (Fabregat et al., 2017), WikiPathways (Slenter et al., 2017)] to identify pathways enriched with the gene set extracted from the entire Heme knowledge map. The enrichment analysis evaluated the overrepresentation of the genes present in HemeKG for each of the pathways in the three aforementioned databases using Fisher's exact test (Fisher, 1992). Furthermore, Benjamini–Yekutieli method under dependency was applied to correct for multiple testing (Yekutieli and Benjamini, 2001). Manual inspection of the enrichment analysis results revealed that the Toll-like receptor (TLR) signaling pathway was the most enriched pathway in Reactome and WikiPathways, and the third most enriched in KEGG (**Supplementary Table S1**). Therefore, this pathway was selected for study in the subsequent investigation.

First, the three different representations of this pathway were downloaded from each database and converted to BEL using PathMe (Domingo-Fernández et al., 2019). Next, the three BEL networks were combined with the HemeKG network highlighting their overlaps (**Supplementary Figures S1**, **S2**) in order to specifically analyze these parts of the combined network. Finally, five experts in the field reconstructed the hypothesized pathways from the combined network. The hypothesized pathways were depicted following the guidelines for scientific communication of biological networks outlined by Marai et al. (2019).

RESULTS

Building a Mechanistic Knowledge Graph Around Heme Biology in the Context of Hemolytic Disorders

We introduce the first knowledge graph made publicly available to the biomedical and bioinformatics community focused on heme biology (**Figure 2**). The presented heme knowledge graph was based on the selection of 40 original research articles and 6 review articles related to heme and its role in several pathways. These pathways include the tumor necrosis factor (TNF) and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) signaling pathways, and the complement and coagulation cascades, through which heme plays a role in hemolysis, inflammation and thrombosis (Dutra and Bozza, 2014; L'Acqua and Hod, 2014; Roumenina et al., 2016; Martins and Knapp, 2018; Vogel and Thein, 2018). The focus of the review articles was chosen because of the relevance of these diseases and complications to large numbers of patients (L'Acqua and Hod, 2014; Litvinov and Weisel, 2016; Roumenina et al., 2016; Effenberger-Neidnicht and Hartmann, 2018). All of these pathologies are known to be interconnected and mapping them in relation to heme is promising for the discovery of yet overlooked links.

Following the guidelines outlined in the Methods section, knowledge was manually extracted and encoded from each of these 46 articles using BEL because of its ability to represent not only causal, but also correlative and associative relationships found in the literature, as well as corresponding provenance and experimental contextual information. This curation exercise resulted in HemeKG, a knowledge graph containing 775 nodes (**Table 1**) and 3,051 relations (**Table 2**), as well as contextual information ranging from cellular and anatomical localization to different states of the heme molecule (**Supplementary Figure S1**). Annotations, such as time point and concentration, enabled us to capture time dependencies between entities. By using this contextual information and the multiple biological scales presented in the model, we have not only been able to represent a part of heme's interactome (**Figure 2**), but also established

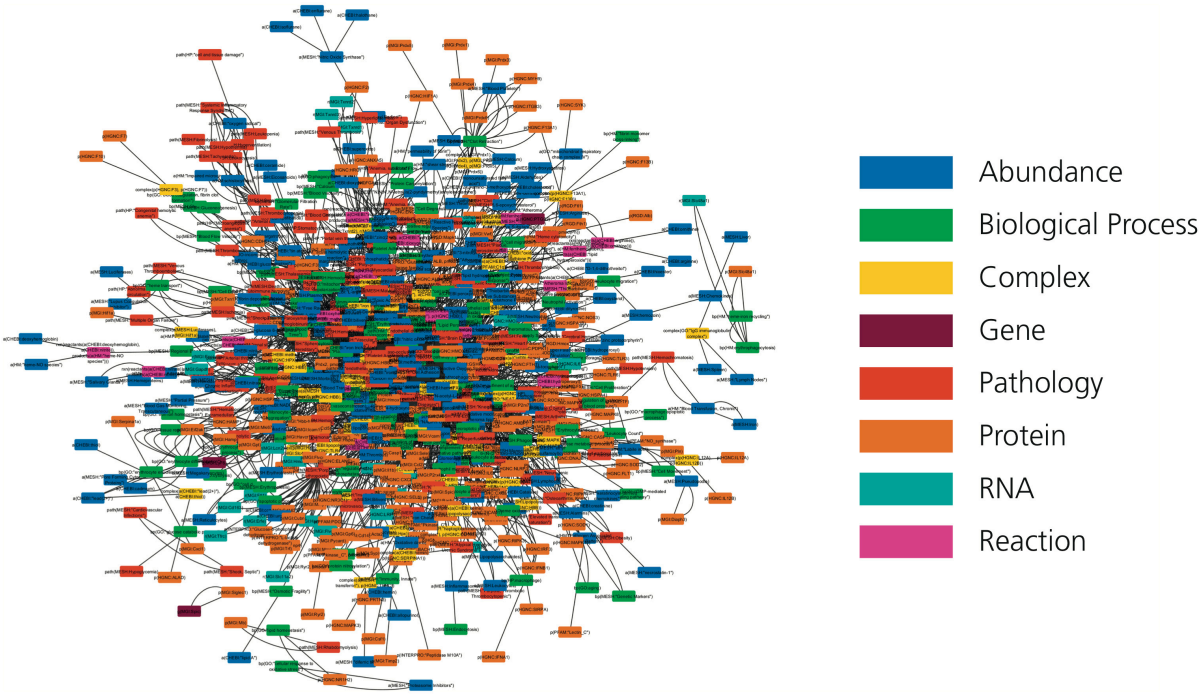

FIGURE 2 | The HemeKG network. Nodes are colored by their different functions in BEL (see legend).

several links to phenotypes and clinical endpoints. Both represent essential considerations for the design of future clinical studies of hemolytic conditions.

Finally, to facilitate the use of the curated content in this work, BEL documents are bundled with a dedicated Python package that enables direct access to the content, provides conversion utilities and allows for network exploration. Both the BEL documents and the Python package are available at https://github.com/hemekg/hemekg.

Curating a Supporting Heme Terminology

The specificity of our work, together with the lack of contextual terminologies related to heme biology, prompted us to generate a supporting terminology focused on heme. It contains more than 50 terms that delineate heme-related entities, such as biological processes, proteins, or pathologies that are not yet included in other standard resources such as (GO Ashburner et al., 2000). Building this terminology not only allowed us to describe entities with more expressiveness, but also facilitates text mining or annotation tasks related to the heme molecule in the future. The terminology is available at https://github.com/hemekg/terminology.

Dissection of the Crosstalk Between Heme and TLR Using HemeKG

The established heme knowledge graph can be used to study the crosstalk of heme biology with a pathway of interest. HemeKG is of special interest in the context of hemolytic disorders, such as malaria and sickle cell anemia, because these diseases are associated with the release of heme into circulation. Heme can then exert a detrimental role by regulating several proteins and signaling pathways (Kühl and Imhof, 2014). In order to select a pathway that highly overlaps with the generated network, we conducted pathway enrichment analysis using three major databases (i.e., KEGG, Kanehisa et al., 2016), Reactome (Fabregat et al., 2017), and WikiPathways (Slenter et al., 2017). The results of the enrichment analysis in the three databases pointed to TLR signaling as the most enriched pathway (**Supplementary Table S1**). Thus, we proceeded to analyze the crosstalk between this pathway and heme biology by exploring the overlap between HemeKG and the TLR pathways in the three aforementioned databases. Although heme has been linked to numerous (TLRs) including TLR2, TLR3, TLR4, TLR7, and TLR9 (Figueiredo et al., 2007; Lin et al., 2010; Dutra and Bozza, 2014; Min et al., 2017; Merle et al., 2019; Sudan et al., 2019), our analysis was prioritized on the most well-documented interaction, the one between heme and TLR4. Heme stimulates TLR4 to activate NF-κB secretion via myeloid differentiation primary response 88 (MyD88)-mediated activation of lκB (IKK) (see below). Activated IKK promotes the proteolytic degradation of NFKBIA. The phosphorylated IKK complex indirectly activates NF-κB and mitogen-activated protein kinases, such as JNK (C-Jun N-terminal kinase), ERK, and p38 leading to the secretion of TNF-α, interleukin 6 (IL6), IL1B, and keratinocyte-derived chemokine (Dutra and Bozza, 2014). This finally results in an activation of the innate immunity and the generation of proinflammatory factors, which reflects the relevance of heme in several disorders comprising inflammation and infection.

TABLE 1 | Summary of unique nodes for each entity class.

Abundances	Genes	RNAs	Proteins	Complexes	Reactions	Pathologies	Biological processes	Total
200	4	25	226	54	17	128	121	**775**

Each entity class corresponds to the terms formalized in BEL (more information at https://language.bel.bio).

TABLE 2 | Summary of relationship classes.

Increase	Decrease	Positive correlation	Negative correlation	Has component	Association	Causes No change	Ontological relations	Total
639	380	1,322	440	113	54	39	64	**3,051**

Each class corresponds to the relationships formalized in BEL (more information at https://language.bel.bio). The ontological relations class includes the following relationships: has reactant, has product, and has variant.

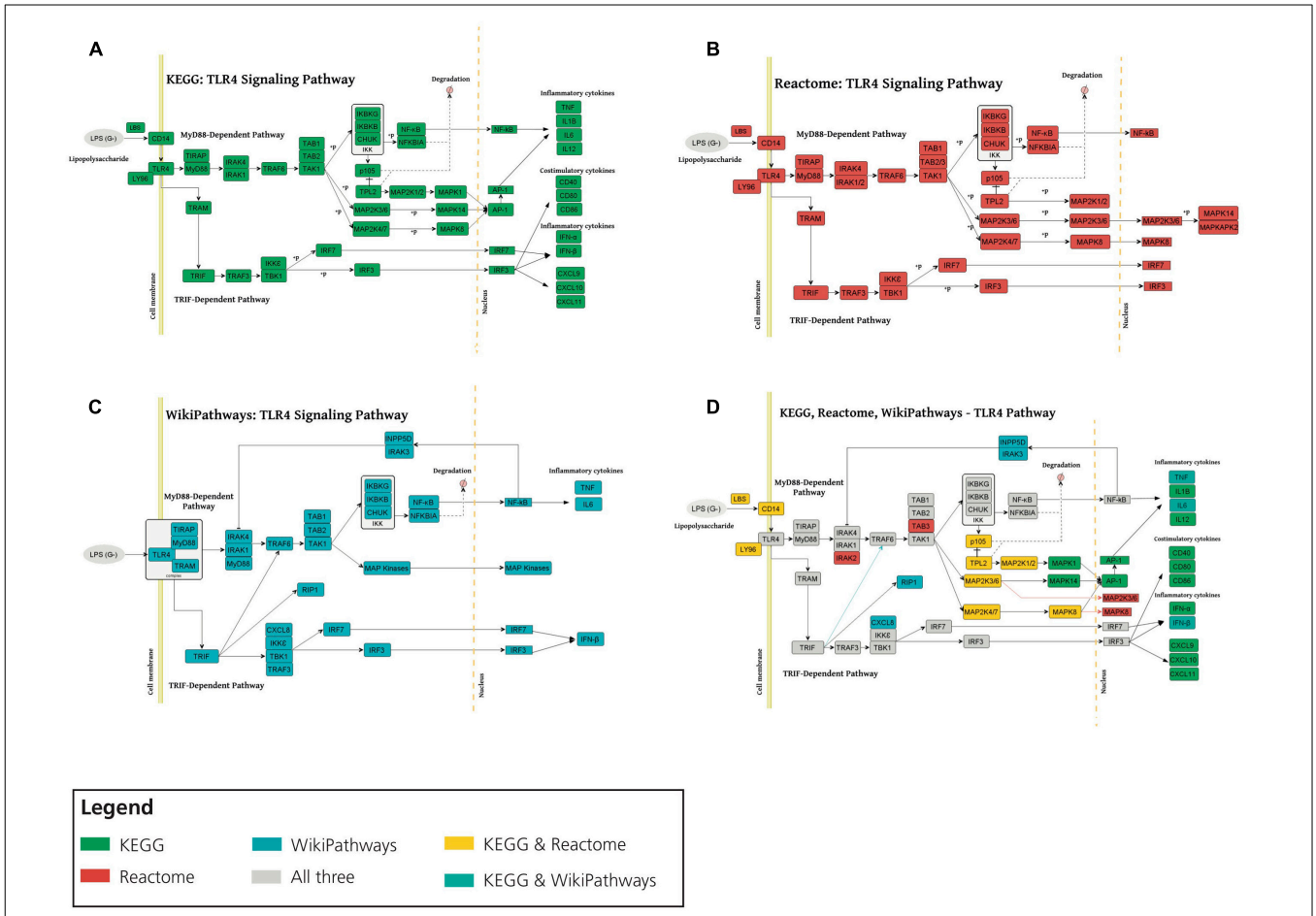

FIGURE 3 | Consensus around the TLR4 signaling pathway in three major pathway databases. TLR4 signaling pathway visualization of KEGG **(A)**, Reactome **(B)**, and WikiPathways **(C)**. **(D)** Superimposing TLR4 signaling pathway from KEGG, Reactome and WikiPathways. Each color corresponds to the presence of the given node in one or multiple databases (see Legend). MyD88, TAK1, IKK complex, MAP kinases, TNF, NF-κB, TRIF and IRF3 emerged in all three databases and also in HemeKG. KEGG and Reactome showed identical representations of the TLR4 pathway whereas WikiPathways was different in a way that nuclear NF-κB activates INPP5D-IRAK3 (inositol polyphosphate-5-phosphatase D IL1 receptor associated kinase 3) complex which inhibits the activity of IRAK1/IRAK4 (IL1 receptor associated kinase 1/4).

We first investigated the consensus of the three different representations of the TLR4 signaling pathway (**Figure 3A**). We observed that, overall, all three representations share a high degree of consensus as illustrated in **Figures 3B–D**. Here, we would like to point out that while KEGG and Reactome present practically identical representations, the WikiPathways

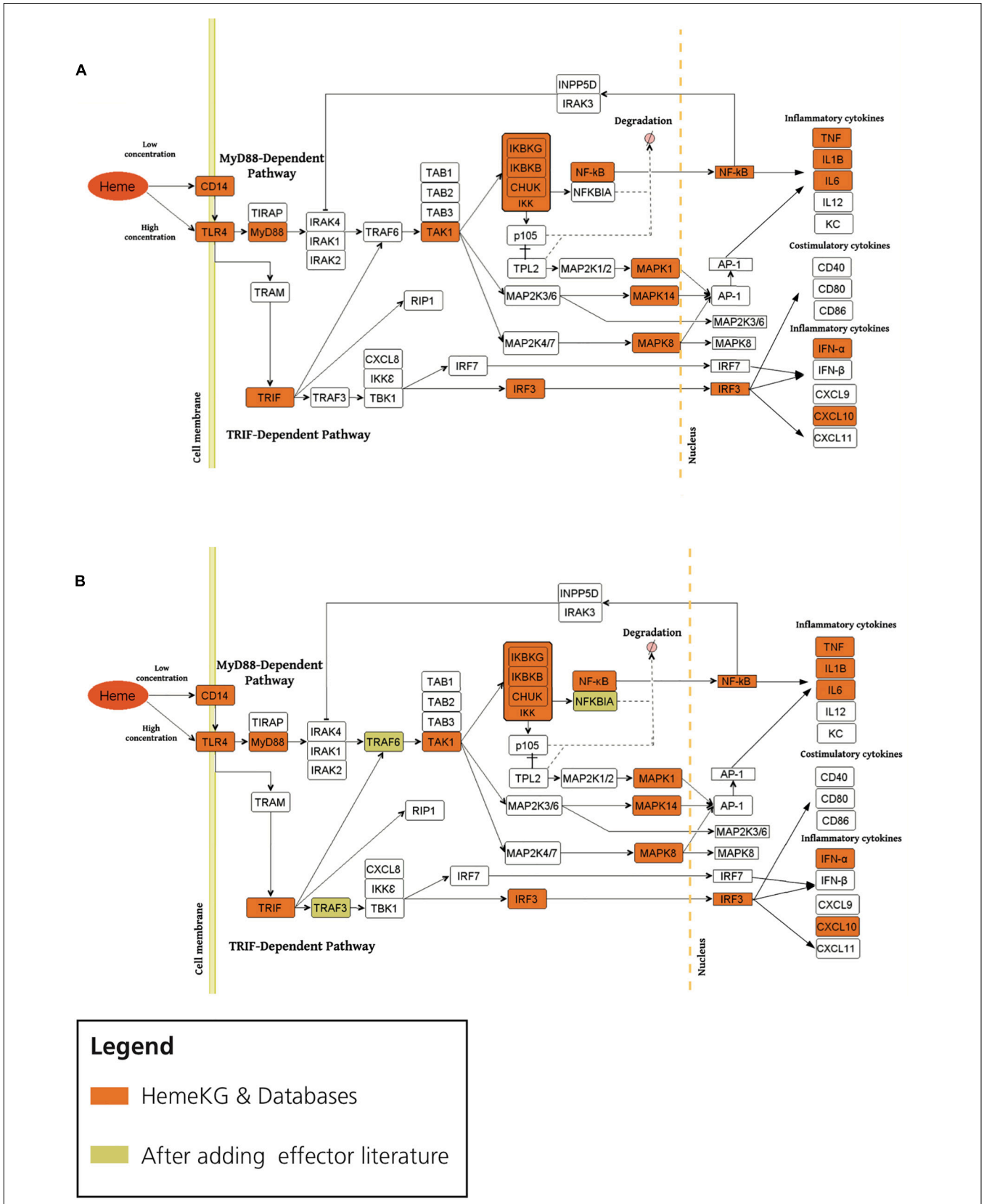

FIGURE 4 | Continued

FIGURE 4 | Overlaying the consensus TLR4 signaling pathway in databases with HemeKG (**A:** original overlaid network, **B:** overlaid network after inclusion of literature evidence for effectors). The orange colored boxes display the common effector molecules between the canonical TLR4 signaling pathway and induced TLR4 signaling pathway stimulated by labile heme. Heme/TLR4 activates the adaptor molecule MyD88. Activated MyD88 promotes the degradation of NFKBIA (NF-κB inhibitor α) through phosphorylation of the IKK complex (inhibitor of nuclear factor κB kinase complex), thus promoting NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) and MAPKs (mitogen-activated protein kinases) stimulation leading to the secretion of TNF-α, IL6, IL1B and KC (keratinocyte-derived chemokine) (Fortes et al., 2012; Dutra and Bozza, 2014). The TRIF (Toll-like receptor adaptor molecule 1) dependent pathway is activated upon signaling of heme through TLR4 leading to the activation of IRF3 (interferon regulatory factor 3) stimulating the secretion of interferons (i.e., IFN-α) and CXCL10 (C-X-C motif chemokine ligand 10) (Dickinson-Copeland et al., 2015). However, the activation profiles for IRAK1/2, TRAF6, TRAM, TRAF3, TBK1/IKK epsilon complex and IRF7 are not yet studied for heme-TLR4 signaling pathway.

representation exhibits slight differences. These differences and complementarities between pathways provide us with a more comprehensive view of the studied pathways, as illustrated by our previous work (Domingo-Fernández et al., 2019).

Second, in order to study the overlap between TLR4 signaling pathway and heme biology, we overlaid the consensus network of the pathway with HemeKG (**Figure 4**). Superimposing both networks revealed that MyD88, TAK1, IKK complex, MAP kinases, TNF, NF-κB, Toll-like receptor adaptor molecule 1 (TRIF), and interferon regulatory factor 3 (IRF3) were present in all three databases and in our model. However, several effector molecules, which were found in the three databases, were not found in our heme knowledge graph (HemeKG), for example, IL1 receptor-associated kinase proteins 1, 2, and 4 (IRAK1, IRAK2, and IRAK4, respectively); TNF receptor-associated factor 6 (TRAF6); TAB1-3; and others (**Figure 4A**). Thus, we specifically searched for literature reports of these effectors in the context of heme signaling, by entering the respective queries in PubMed, as this knowledge might not have been sufficiently covered by the 40 original research articles selected to establish HemeKG.

The activation profile for labile heme as an extracellular signaling molecule through TLR4 was suggested to be similar to the one established via Lipopolysaccharides (LPS) as signaling molecule from standard pathway databases (Pålsson-Mcdermott and O'Neill, 2004). This pathway begins with the induction of TIRAP (Mal)-associated MyD88 signaling on the one hand (Horng et al., 2002), and TRAM (TICAM-2)-associated TRIF (TICAM-1)-signaling, on the other hand (Seya et al., 2005), resulting in the upregulation of proinflammatory cytokines and chemokines (**Figure 4**). MyD88 protein as an adaptor has been shown to interact with IL1 receptor-associated kinase (IRAK) proteins 1, 2, and 4 to start the signaling cascade involving TRAF6, which is known to activate IKK in response to proinflammatory cytokines. However, in our heme knowledge graph the connections between IRAKs, TRAF6, and TAB proteins were missing (**Figure 4A**). By taking a closer look at these effectors in the context of heme, we found various information for example TRAF6 indicating both a direct and indirect link to heme-induced signaling via TLRs (Hama et al., 2012; IJssennagger et al., 2012; Park et al., 2014; Huang et al., 2015; Meng et al., 2017). In contrast, other effector molecules such as IRAK and TAB proteins (**Figure 4**) were not described in heme signaling so far. We then performed a PubMed search for these missing terms in combination with "heme." These findings led us to refine HemeKG so that only those signaling components for which no evidence was found manually still remain as white spots on the map (**Figure 4B**).

In addition, the preceding discussion has excluded parameters such as the concentration of labile heme available in the respective environment. This aspect will be particularly important, if heme-triggered signaling pathways are dependent on, or determined by the concentration of heme. At lower concentrations of heme, TLR4 signaling has been described to be CD14 dependent, whereas at high concentrations of heme, TLR4 activation does not require CD14 (Piazza et al., 2010; **Figure 4**). Also, there is a need to further investigate whether heme/TLR4 induction of the adapter molecule MyD88 is dependent or independent of TIRAP activation, similar to the LPS/TLR4 induced TIRAP-associated MyD88 signaling pathway. Furthermore, heme/TLR4 activates a pathway that leads to the activation of IRF3, resulting in the production of interferons for example, IFN-α (Dutra and Bozza, 2014) and overproduction of C-X-C motif chemokine 10 (CXCL10) (Lin et al., 2012; Dickinson-Copeland et al., 2015). In the literature, the molecular mechanism by which heme/TLR4-induced TRAF3 and IRF3/7 activation leads to the secretion of IFN-α and CXCL10 is not represented. It is therefore shown as a white box in the map (**Figure 4B**). Finally, the introduction of noncanonical pathways and receptor crosstalk-triggered cascades go beyond the scope of this work, but represent opportunities for future studies on heme signaling.

DISCUSSION

We have presented HemeKG, the first mechanistic model in the context of heme biology, as a viable solution to comprehensively summarize heme-related processes by bringing knowledge from disparate literature together. Furthermore, we have demonstrated how combining the knowledge from the heme knowledge graph with information available in pathway databases provides new insights into the network of interactions that regulate heme pathophysiology.

Because HemeKG was curated using standard vocabularies, its content can be linked to the majority of public databases. Therefore, enriching the HemeKG network with external data or incorporating its integrated knowledge into other resources is feasible. For example, the entire Bio2BEL framework[1] can be used to scale up this resource by enriching HemeKG with dozens of widely used biomedical databases. In order to make HemeKG accessible to a wider audience, we uploaded it to BEL Commons - a web application for curating, validating, and exploring knowledge assemblies encoded in BEL

[1]https://github.com/bio2bel

(Hoyt et al., 2018). Users can interactively explore the network, make modifications, integrate additional resources via Bio2BEL, and share those modifications using its versioning system. Furthermore, the variety of formats that our resource can be converted to also facilitates its use by other systems biology tools such as Cytoscape (Shannon, 2003) and NDEx (Pratt et al., 2015). In summary, the characteristics of HemeKG make this resource suitable not only for hypothesis generation as presented in our case scenario, but also for clinical decision support as previously demonstrated with other systems biology maps (Ostaszewski et al., 2018). For instance, computational mechanistic models are currently being used in combination with artificial intelligence methods for a variety of predictive applications (Khanna et al., 2018; Esteban-Medina et al., 2019; Çubuk et al., 2019). Instead of contextless canonical pathways as until now (i.e., pathways describing normal physiology), HemeKG could be used for predicting drug response and for drug repurposing in numerous related disorders such as malaria and sepsis. Finally, the supporting terminology built during this work could be used for a broad range of applications from data harmonization to natural language processing.

A potential limitation of this study is that it is constrained to a specific literature corpus as we are aware that the presented knowledge graph captures only a part of a much larger interaction network. This tends to be a common challenge when constructing contextualized maps and is further compounded by the difficulty in assessing the coverage of a network, explaining why some nodes are missing in HemeKG compared to the three pathway databases used in this study. Furthermore, the bias in the scientific community against publishing negative results must also be acknowledged. A clear example is how the hypotheses of our crosstalk analysis could be complemented by this knowledge gap that could reveal new interesting hypotheses. Thus, future updates in HemeKG, as in any work of this kind, will be required while prioritizing time and effort (Rodriguez-Esteban, 2015). Further, advanced network-based analyses (Catlett et al., 2013) could be used to rank heme-related pathways in the context of a given *-omics* data set.

Although numerous interactions between heme and TLRs have been described in the literature (Lin et al., 2010; Min et al., 2017), their downstream effects have not been contextualized (i.e., presented in a coherent/integrated manner like a knowledge model does). The analysis we have presented focusing on the crosstalk between heme biology and the TLR signaling pathway has shed some light on how this crosstalk could be related to heme biology. However, there also exist other well-known pathways related to heme, that could be investigated by conducting similar analyses in the future.

DATA AVAILABILITY STATEMENT

The data sets and scripts of this study can be found at https://github.com/hemekg.

AUTHOR CONTRIBUTIONS

DI, MH-A, and DD-F conceived and designed the study. FH curated the data and conducted the main analysis supervised by AP, DI, and DD-F. M-TH, BS, MD, and AP assisted in selecting the corpora and interpreting the results. CH designed the curation guidelines and implemented the Python package. DD-F, FH, CH, M-TH, BS, MD, and DI wrote and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.00074/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4.3.2 Summary

For the first time, the network of heme-triggered effects in hemolytic disorders was depicted in a knowledge graph, called HemeKG. Starting from six review and 40 original articles that deal with the consequences of heme in hemolytic states, reported causal and correlative relationships were encoded and used for the generation of HemeKG. In total, HemeKG contains 775 nodes (mainly proteins, pathologies and biological processes with 226, 128, and 121 nodes, respectively) and 3051 relations. Besides hemolysis, thrombosis and inflammation emerged as main nodes. Upon pathway enrichment analysis with common pathway databases, TLR4 signaling appeared as the most pronounced pathway. Superimposition of the database-derived TLR4 signaling pathway and HemeKG revealed several effector molecules that are already known to be affected by heme (such as MyD88, MAPKs, and TNFα), bringing them into a causal relation network. Furthermore, others, like the Toll/IL-1 receptor domain-containing adapter protein (TIRAP) and several IL-1 receptor-associated kinase proteins (IRAKs), were not linked with heme so far.

Hence, together with the identification of so far unknown heme-regulated proteins, the herein applied knowledge-based modeling approach provides a valuable tool for the analysis of heme's effects under hemolytic conditions on the molecular basis with further (so far not noticed) starting points for future research and possibly the development of approaches for the treatment of heme-driven pathologies, including proinflammatory and prothrombotic complications.

4.4 Unravelling the debate on heme effects in COVID-19 infections

Original research article

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4.4.1 Introduction

The current pandemic is caused by infections with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), leading to COVID-19. Upon entry into the lungs via a protein-mediated mechanism, the virus triggers vascular dysfunction, finally leading to pneumonia and acute respiratory distress syndrome along with a rise of proinflammatory cytokines (cytokine storm) and markers of hypercoagulability.[458] Furthermore, a decline of hemoglobin and an increase in hemopexin levels has been reported, suggesting a potential link to heme pathology.[459] Thus, a direct role of heme in COVID-19 pathology might be conceivable through parallel occurring hemolytic events or a preexisting comorbidity, which might further enhance disease progression. To address this, two approaches are presented in the following. On the one hand, proteins that participate in the virus entry process are analyzed for the heme-binding ability. In addition, an overlay between the knowledge graph on heme pathology (HemeKG) and a similar knowledge graph for COVID-19 pathology (COVID-19 KG[453]) aims for the detailed analysis of parallels in induced pathways and resulting changes on molecular basis.

Unravelling the debate on heme effects in COVID-19 infections

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Abstract

The SARS-CoV-2 outbreak was recently declared a worldwide pandemic. Infection triggers the respiratory tract disease COVID-19, which is accompanied by serious changes of clinical biomarkers such as hemoglobin and interleukins. The same parameters are altered during hemolysis, which is characterized by an increase in labile heme. We present two approaches that aim at analyzing a potential link between available heme and COVID-19 pathogenesis. Four COVID-19 related proteins, i.e. the host cell proteins ACE2 and TMPRSS2 as well as the viral protein 7a and S protein, were identified as potential, though differing heme binders. We also performed a detailed analysis of the common pathways induced by heme and SARS-CoV-2 by superimposition of knowledge graphs covering heme biology and COVID-19 pathophysiology. Herein, focus was laid on inflammatory pathways, and distinct biomarkers as the linking elements. Our results contribute to the understanding of the progression of COVID-19 infections in patients with different clinical backgrounds and might allow for a more individual diagnosis and therapy in the future.

1 Introduction

In the beginning of 2020, the **co**rona**vi**rus **d**isease **19** (COVID-19) has been declared a pandemic of international concern and an unprecedented challenge for the country-specific health care systems (Cucinotta and Vanelli, 2020). COVID-19 is caused by infections with **s**evere **a**cute **r**espiratory **s**yndrome **co**rona**v**irus **2** (SARS-CoV-2) and is accompanied by pneumonia, acute respiratory distress syndrome (ARDS) associated with a cytokine storm, and death in the most severe cases (Ragab et al., 2020; Ye et al., 2020; Zhou et al., 2020a). By taking a closer look into the molecular mechanisms of the infection and disease development, it is important to note that patients with severe COVID-19 often had a history of hypertension, yet also chronic kidney disease, cardiovascular disease or diabetes mellitus compared to those with milder disease progression (Ji et al., 2020; Mantovani et al., 2020; Zhou et al., 2020a). Among other criteria, also the patients' blood group seems to affect the disease progression (Latz et al., 2020; Li et al., 2020; Wu et al., 2020; Zhao et al., 2020). However, the scientific evidence of a potential higher risk for these patients, is still pending. Furthermore, there is evidence that the renin-angiotensin system (RAS), which is associated with hypertension, is directly associated with viral transmission (Hanff et al., 2020; Mascolo et al., 2020; Yi et al., 2020). An essential part of RAS is the enzyme angiotensin-converting enzyme 2 (ACE2) that is expressed on the cell surface of alveolar epithelial cells of the lungs (Ren, 2006). More precisely, a recent report identified specific bronchial transient secretory cells, a cell state between goblet (responsible for mucus production) and ciliated cells (responsible for airway clearance) in human bronchial epithelial cells to be primarily attacked during the viral infection (Lukassen et al., 2020; Xu et al., 2020).

The virus gains access to the host cell by docking of its spike proteins (S protein) to the membrane surface of the host cell, which primarily occurs via the transmembrane protein ACE2 (Ali and Vijayan, 2020; Hoffmann et al., 2020). This interaction between the host cell and SARS-CoV-related viruses is known since 2012 and involves in case of SARS-CoV-2 several residues in the receptor-binding domain of the S protein and several residues of ACE2 that form hydrogen bonds and an hydrophobic interaction interface as well as a salt bridge between Asp40 (ACE2) and Lys417 (S protein) (Wu et al., 2012; Yi et al., 2020, Zhai et al., 2020). In this context, the membrane protein M (M protein) is discussed to be relevant for the entry and attachment of the virus by interacting with the S protein (Bianchi et al., 2020). Furthermore, the M protein may also be important for the budding process of the virus, since it interacts with the nucleocapsid envelope protein (E protein) and the S protein during virus particle assembly (Alsaadi and Jones, 2019). Additionally, it has been proposed that the E protein oligomerizes to form ion channels, and also plays a role in the assembly of the viral genome (Ruch and Machamer, 2012). Although protein 7a has not yet been fully characterized it is already known to act as an accessory protein (as derived by similarity from SARS-CoV), while interacting with M and E proteins (Fielding et al., 2006). This process is essential for virus particle formation before release of the reproduced virus particles into the surrounding areas, such as the blood stream (Fielding et al., 2006; Kwon et al., 2020). Recent studies have revealed that the affinity of SARS-CoV-2 for ACE2 is 10-20-times higher than the affinity of SARS-CoV, which would explain its much higher transmissibility (Hoffmann et al., 2020; Zhang et al., 2020b). Upon binding, the viral S protein is subjected to proteolytic cleavage by the host cell's transmembrane serine protease subtype 2 (TMPRSS2) (Hoffmann et al., 2020). The virus' entry can be blocked by a clinically proven protease inhibitor, rendering the TMPRSS2 an interesting target against COVID-19 (Hoffmann et al., 2020). Interestingly, it was shown that SARS-CoV-2 does not use other receptors like aminopeptidase N or dipeptidyl peptidase 4 for cell entry as described for other coronaviruses (Zhou et al., 2020b). Therefore, these proteins are unlikely to represent suitable targets for therapy of COVID-19.

The tissue distribution of one of the main actors, ACE2, in organs like heart, kidney, endothelium, and intestine might explain the multi-organ dysfunction observed in COVID-19 patients (Zhang et al., 2020b). Several studies have provided information about the main symptoms, risk factors for severe

disease progression, and clinical diagnostic values including blood routine, blood biochemistry, and infection-related biomarkers (Chen et al., 2020; Guan et al., 2020; Han et al., 2020; Zhou et al., 2020a). Although the details of these individual studies vary, there is a consensus among changes of numerous clinical parameters, which might be directly connected or must be considered together in a specific context. For example, hemoglobin is decreased in more than 50% of the patients, as is serum albumin in 98% (Chen et al., 2020; Guan et al., 2020; Whetton et al., 2020; Yang et al., 2020; Zhou et al., 2020a). In intensive care unit (ICU) patients, reduced levels of hemoglobin levels and cluster of differentiation proteins (CD) 45, CD4, CD8, CD19, and CD16/56 were observed (Fan et al., 2020). In contrast, values of absolute lymphocyte count and absolute monocyte count were comparable to non-ICU patients (Fan et al., 2020). However, in contrast to human immunodeficiency virus (HIV) and cytomegalovirus, the CD4/8 ratio was not inverted (Fan et al., 2020). Main symptoms are fever, cough, and fatigue, all presenting reactions of an activated immune system (Guan et al., 2020). The activation of the immune and the complement system is also observed by a variety of markers including increased values for interleukin (IL)-6 (52% of the patients), erythrocyte sedimentation rate (85%), serum ferritin (63%), and C-reactive protein (CRP) (86%) (Chen et al., 2020; Risitano et al., 2020; Wang et al., 2020; Whetton et al., 2020; Zhang et al., 2020c; Zhou et al., 2020a). Furthermore, recent studies that monitored and compared coagulation parameters of COVID-19 patients have suggested a tendency to procoagulant states (Han et al., 2020; Ji et al., 2020; Tang et al., 2020) and an increased risk of venous thromboembolism (Giannis et al., 2020), which was indicated by higher levels of fibrin/fibrinogen degradation products and fibrinogen itself, as well as lower antithrombin levels (Han et al., 2020). Moreover, D-dimer levels, a marker for coagulation and sepsis, are markedly increased in non-survivors of COVID-19 (Guan et al., 2020; Zhou et al., 2020a). Therefore, COVID-19 patients often suffer from leukopenia, lymphocytopenia, and thrombocytopenia (Guan et al., 2020).

Overall, these clinical parameters are interrelated when viewed from the perspective of heme and its interaction radius (Dutra and Bozza, 2014; Kühl and Imhof, 2014; Roumenina et al., 2016; Humayun et al., 2020). Heme is well-known as the prosthetic group of diverse proteins, e.g., hemoglobin, where it is responsible for the oxygen transport in the blood (Ascenzi et al., 2005). Under hemolytic conditions such as malaria, sickle cell anemia (SCD), β-thalassemia, and hemorrhage, or in case of severe cellular damage, heme is released in enormous amounts as a result of hemoglobin degradation, leading to a pool of labile heme (Ascenzi et al., 2005; Roumenina et al., 2016). In this case, the heme-detoxifying scavenger proteins, in particular hemopexin, become saturated, which allows heme to execute its wide-ranging effects (Chiabrando et al., 2014; Roumenina et al., 2016; Detzel et al., 2020). The response to heme in this context leads to cytotoxic, procoagulant, vasculotoxic, and proinflammatory effects, as well as an activation of the complement system (Dutra and Bozza, 2014; Roumenina et al., 2016). Labile heme also plays a central role in the pathology of severe sepsis which leads to vascular inflammation and severe toxic effects in organs like liver, kidney or cardiac tissue (Dutra and Bozza, 2014). These responses are, in part, mediated through direct interaction of heme with the responsible proteins (i.e. tumor necrosis factor α (TNFα), Toll like receptor 4 (TLR4), and complement factor 3 (C3) (Dutra and Bozza, 2014; Roumenina et al., 2016; Kupke et al., 2020), or by up-regulation of the respective cytokines, including IL-1β, IL-6, and TNFα (Dutra and Bozza, 2014). In addition, for heme a downstream ROS-dependent induction of distinct signaling pathways (MAPK/ERK pathway, NFκB signaling) is discussed, which can lead to stimulation of neutrophil recruitment, necrotic cell death or expression of adhesion molecules (Dutra and Bozza, 2014). In fact, we recently contextualized the role of heme as an inflammatory mediator as well as its crosstalk with the TLR4 signaling pathway (Humayun et al., 2020).

Although the aforementioned findings suggest a connection between biological processes implicated in SARS-CoV-2 and those related to heme, there is a lack of information on both subject areas. Despite a considerable volume of research on SARS-CoV-2 over the past few months, knowledge of the molecular mechanisms responsible for the pathophysiology of the virus still remains scarce. Likewise,

data in the context of heme's biology is underrepresented, if available at all, in standard bioinformatics resources such as pathway databases. This knowledge-gap can be addressed using custom-made models, namely Knowledge Graphs (KGs). KGs can be built by extracting logical relations from research manuscripts (Kodamullil et al., 2015). In this fashion, the available knowledge is integrated and brought into a broader context. Placing information in the right context can lead to the identification of biochemical mechanisms, hypothesis generation, and targets for drug repurposing (Kodamullil et al., 2015; Karki et al., 2017; Emon et al., 2017). Most importantly, KGs are ideally suited for tracing common pathways in different diseases, as we have shown for type II Diabetes mellitus and Alzheimer's disease (Karki et al., 2020). Two of our recent scientific publications have focused on KGs around heme as well as SARS-CoV-2 (Domingo-Fernández et al., 2020; Humayun et al., 2020). Although both studies are tangential, tthe generated KGs can subsequently be employed to investigate the overlap between hemolytic disorders and COVID-19. By integrating the COVID-19 and Heme KGs we aim to shed light on the shared mechanisms between these two possibly interdependent domains.

Following the deposition of a not yet peer-reviewed manuscript stating that SARS-CoV-2 attacks hemoglobin 1-β chain and captures the porphyrin of its heme (Liu and Li, 2020), a heated debate arose about the scientific substantiation of the truthfulness of the claims. In this context, one should ask: How realistic is it, in view of the patients' constitution that coronavirus components encounter heme and following up on this, what might be the consequences of such an interaction, if any? Thus, in order to contribute to the understanding of SARS-CoV-2, its strategies of infection, as well as the pathogenesis and the course of disease in coronavirus patients, we combined our know-how and expertise for a joint analysis of the aforementioned issue. On the one hand, we examine the possibility of a direct interaction of heme with select SARS-CoV-2 proteins and specific host cell proteins by applying our webserver HeMoQuest (Paul George et al., 2020) that is based on experimental data. One of the most promising findings was the prediction of heme-binding motifs (HBMs) in the host cell proteins ACE2 and TMPRSS2. On the other hand, by superimposing the two knowledge graphs, i.e. heme KG (Humayun et al., 2020) and COVID-19 KG (Domingo-Fernández et al., 2020), we provide insights into pathways that might play a role when considering heme in the context of COVID-19 infections. Finally, our results suggest that proinflammatory pathways could connect the pathophysiology of elevated heme with COVID-19 disease progression.

2 Materials and Methods

2.1 Screening for potential heme-binding motifs in COVID-19 related proteins

HeMoQuest (http://131.220.139.55/SeqDHBM/) (Paul George et al., 2020) was used to identify potential HBMs in the following proteins of SARS-Cov-2: S protein, M protein, E protein, and protein 7a, and human: ACE2 and TMPRSS2. For the selection, the procedure described earlier was applied (Wißbrock et al., 2019; Detzel et al., 2020; Hopp et al., 2020).

2.2 Synthesis, purification and amino acid analysis of protein 7a- and ACE2-derived peptides

To further substantiate the screening with experimental data, the potential HBMs of protein 7a and ACE2 were synthesized as nonapeptides (2 protein 7a-derived peptides, 5 ACE2-derived peptides), as described before (Kühl et al., 2011; Kühl et al., 2013; Brewitz et al., 2015; Brewitz et al., 2016). In brief, automated solid-phase peptide synthesis was performed by standard Fmoc/tBu strategy on Rink amide MBHA resin. Preparative reverse-phase HPLC (PU-987, JASCO) was used to purify the crude products. Subsequently, the peptides were characterized by analytical HPLC (LC-20A, Shimadzu) and either by LC-ESI-MS (micrOTOF-Q III, Bruker Daltonics; UltiMate 3000 LC, ThermoScientific) or

MALDI-TOF-MS (UltrafleXtreme, Bruker Daltonics) (Table S1). Moreover, for the determination of the peptide content amino acid analysis was carried out (LC3000, Eppendorf-Biotronik).

2.3 Heme binding analysis of protein 7a- and ACE2-derived peptides with UV/vis spectroscopy

As described earlier (Kühl et al., 2013), the heme-binding capacity of the 7 peptides was evaluated by UV/vis spectroscopy. Heme (0.4 - 40 µM) was incubated with each peptide (10 µM) for 30 min in HEPES buffer (100 mM, pH 7.0). Afterwards, absorbance spectra over a wavelength range of 300 nm to 600 nm were measured with a Multiskan GO spectrophotometer (Thermo Fisher Scientific). Subsequently, difference spectra were generated through spectra subtraction of the single heme and peptide spectra from the heme-peptide complex spectra. Dissociation constants (K_D) were calculated by using GraphPad prism 8.0 and the already earlier applied equation (Pîrnau et al., 2008; Kühl et al., 2013).

2.4 Homology modeling

In all *in silico* experiments, we used available cryogenic electron microscopy (cryoEM) structures or homology models (publicly available or in-house built). In case of ACE2 the recently published, fully glycosylated cryoEM structure (PDB: 6M18) was used, which was recorded in complex with sodium-dependent neutral amino acid transporter B (B^0AT1) (Yan et al., 2020). B^0AT1 was removed in order to focus on ACE2 only. Although S protein cryoEM structures for open and closed states are available (PDB: 6VXX and 6VYB) (Walls et al., 2020), these structures lack several surface-exposed sequence stretches, in which some of the predicted motifs are located. We therefore used the structure available from the C-I-TASSER structure prediction server reported earlier (Zhang et al., 2020a). For SARS-CoV-2 protein 7a and TMPRSS2, no structures are available so far. Thus, homology models were built using YASARA version 19.12.14 and the *hm_build* macro with default settings (Krieger and Vriend, 2014). We were able to build a hybrid model of the virion surface-exposed part of protein 7a from two structures of the SARS virus protein 7a (PDB: 1XAK and 1YO4). The model achieved an overall Z-score of -0.053, which can be regarded as suitable. In contrast, a hybrid model of TMPRSS2 based on kallikrein and hepsin (PDB: 6I44, 6O1G, 1Z8G, 5CE1) exhibited only a poor overall Z-score of -2.363 and was therefore rejected. In this case, further *in silico* analysis was performed with the available Swiss-model O15393 (Waterhouse et al., 2018).

2.5 Modeling the interplay between SARS-CoV-2 and heme

In order to investigate the mechanisms linking SARS-CoV-2 and heme, we exploited the KGs generated in our previous work (Domingo-Fernández et al., 2020; Humayun et al., 2020). We compiled the two KGs encoded in Biological Expression Language (BEL) using PyBEL (Hoyt et al., 2018) directly from their public repositories (i.e. https://github.com/covid19kg and https://github.com/hemekg) and superimposed their interactions onto a merged network. Given the high degree of expressivity of BEL that enables the representation of multimodal biological information, the KGs were not only enriched with molecular information, but also with interactions from the molecular level to phenotypes and clinical readouts. We leveraged this multimodal information to hypothesize the pathways that connect key molecules associated with SARS-CoV-2 and heme to the phenotypes observed in COVID-19 patients.

Since both KGs comprise several thousands of interactions, manually inspecting all relations and evaluating the implication of the crosstalk between COVID-19 and heme is largely infeasible. Accordingly, this analysis primarily focuses on the set of nodes present in both KGs. Prior to this crosstalk analysis, we conducted a one-sided Fisher's exact test (Fisher, 1992) to confirm the

significance of the overlap between human proteins present in each of the KGs (p-value < 0.01). We then manually classified the set of overlapping nodes into four pathways based on their functional role: i) immune response - inflammation, ii) immune response - complement system, iii) blood and coagulation system, and iv) organ-specific diagnostic markers. Finally, upon superimposing the relations between the overlapping nodes from the Heme and the COVID KGs, we analyzed the signature similarities between each of the above mentioned pathways. These relations are summarized in Figure 3 and also shown in Table S2-S5 together with their evidence and provenance information.

In order to validate the hypotheses coming from the KG, we compared the relations emerging from the overlap between the two KGs with experimental data published in the context of COVID-19 (Blanco-Melo et al., 2020). The concordance of the expression patterns in these datasets with each relation shown in Figure 4 is shown in Table S6.

3 Results

COVID-19 progression severely diverges between affected patients with ARDS and other patients, which could even remain asymptomatic. Current research is thus focusing on explaining the reasons for such discrepancy considering the physical conditions and (pre-)existing illnesses of those affected. With regard to the subject of a possible interrelation between COVID-19 and heme, numerous options need to be regarded. First, the earlier claim of an interaction of protoporphyrin IX with SARS-CoV-2 (Liu and Li, 2020) must be questioned, since heme would appear before protoporphyrin IX as a consequence of e.g., hemolytic conditions. Thus, the direct interaction of heme with viral surface proteins, as well as host cell proteins exposed to virus attack, needs to be considered. Second, systemic hyperinflammation follows severe COVID-19 infection. This becomes manifest by an increase in the abundance of numerous cytokines (e.g., IL-2, IL-7, IL-6, TNFα) (Ye et al., 2020), which is indicative of cytokine release syndrome (Huang et al., 2020) and leads to elevated serum biomarkers in patients (e.g., CRP, lactate dehydrogenase (LDH), D-dimer, ferritin) (Chen et al., 2020; Young et al., 2020; Zhang et al., 2020c; Zhou et al., 2020a). Several of these indications, however, were also reported for labile heme occurring in patients with hemolytic disorders (Litalien et al., 1999; Barcellini and Fattizzo, 2015). Therefore, heme as a key player in initiating or mediating distinct processes in connection with a viral infection needs to be considered as well. This can be exemplified with the interaction of heme with e.g., Zika, Chikungunya, and HIV-1 viruses (Gupta et al., 2015; Lecerf et al., 2015; Assunção-Miranda et al., 2016; Neris et al., 2018). In the following, we present our results concerning the potential direct heme interaction with COVID-19-related proteins as well as a detailed analysis of common pathways of excess heme and COVID-19 pathophysiology.

3.1 Heme-binding ability of surface proteins of SARS-CoV-2 and host cells

Numerous interesting target proteins of the virus and host cell surface were linked with pathological effects of SARS-CoV-2, including E protein, S protein, M protein and protein 7a as well as the human proteins ACE2 and TMPRSS2. All proteins contain at least an extracellular, surface-exposed part and are thus accessible for interaction with heme (Hänel and Willbold, 2007; Mendes de Oliveira et al., 2018; Mousavizadeh and Ghasemi, 2020; Walls et al., 2020). This led us to examine these proteins for potential heme-binding sites. It is known that regulatory heme binding occurs on short surface-exposed HBMs. We identified potential HBMs on all target proteins using the recently published, specialized machine-learning algorithm HeMoQuest (Paul George et al., 2020). This algorithm can predict HBMs from primary structure and was trained on a large array of heme-binding peptides. Screening of the amino acid sequences of S protein, protein 7a, ACE2 and TMPRSS2 resulted in 50, 6, 21, and 32 potential HBMs, respectively. M protein and E protein were dismissed as candidates, since no suitable HBMs were found. HBMs, which are part of the transmembrane or intravirion/intracellular domains,

were removed from the selection. In addition, we excluded motifs in which the central coordinating residue was involved in disulfide bonds or where adjacent residues were glycosylated within the actual protein. After this refinement of the hits, we identified 24 motifs in S protein, two in protein 7a, 15 in ACE2, and 14 in TMPRSS2 (Figure 1). These motifs were then manually screened for surface accessibility using the protein structures, or if unavailable, homology models. Consequently, three motifs for S protein, two motifs for protein 7a, five motifs for ACE2 and ten motifs for TMPRSS2 remained and are discussed below (Figure 1). The potential HBMs in S protein are all located in the N-terminal domain (Figure 1A) (Ou et al., 2020). The first occurring sequence FLGV**Y**^{144}**YH**KN may be the most promising HBM, which is based on a YYH motif and further equipped with phenylalanine at P-4, two additional hydrophobic amino acids (Val, Leu), and a net charge of +2, all beneficial for heme binding (Syllwasschy et al., 2020). The following, I**Y**SK**H**^{207}TPIN and L**H**RS**Y**^{248}LTPG, contain a Y/H-based motif with two spacers between the potential coordinating residues, e.g., YXXH, which have been shown to be less favorable for heme binding (Syllwasschy et al., 2020). Nonetheless, both motifs possess a net charge of +2, and several hydrophobic residues, and are thus likely to bind heme with moderate affinity. In protein 7a, only two overlapping motifs were predicted, which is not surprising due to the small size of 121 amino acids (Figure 1B). Both, DGVK**H**^{73}V**Y**QL and VK**H**V**Y**^{75}QLRA, possess a HXY motif (Syllwasschy et al., 2020) and three hydrophobic residues, rendering it a moderate heme binder and, in turn, protein 7a as a less interesting candidate for interaction with heme.

The analysis of ACE2 revealed five HMBs in total, two of which representing promising H/Y motifs (Figure 1C). The most interesting HBM is LTA**HH**^{374}EMG**H** comprising a HXXXH motif, which was recently shown to exhibit high heme-binding affinity (Syllwasschy et al., 2020). The central H^{374} is immediately adjacent to the site that is essential for cleavage by ADAM17 and part of the zinc(II) ion binding site of ACE2 (Towler et al., 2004; Heurich et al., 2014; Lan et al., 2020). The occurrence of three histidines may be favorable for heme binding as could L^{370}, while E^{375} might be slightly detrimental. On protein level, the present zinc(II) ion might abolish heme binding. The second interesting motif is PL**Y**E**H**^{239}L**H**A**Y**, since it contains the efficient HXH motif (Syllwasschy et al., 2020) with further advantageous aromatic tyrosines (Y^{237}, Y^{243}) and hydrophobic leucines (L^{236}, L^{240}). However, the only possible limitation to affinity for both motifs might be E^{375} and E^{238}, respectively. The third motif SFIR**Y**^{515}**Y**TRT has a YY motif. Although such motifs were shown to be less favorable with respect to affinity (Syllwasschy et al., 2020), this motif displays two basic residues that might support rather good heme binding to the motif. The remaining two motifs QAAK**H**^{535}EGPL and AMRQ**Y**^{654}FLKV are less promising because they only contain one coordinating amino acid.

The largest number of motifs, i.e. ten in total, was identified in the transmembrane serine protease TMPRSS2. Four of these motifs contained only one coordinating amino acid. Additional three motifs (CVRL**Y**^{152}GPNF, RKSW**H**^{169}PVCQ, CAKA**Y**^{469}RPGV) contain a cysteine residue which is involved in a disulfide bond within the protein. These motifs might be less favorable. Two further overlapping motifs (KVIS**H**^{334}PN**Y**D and S**H**PN**Y**^{337}DSKT) were found in the protease domain of TMPRSS2 (Figure 1D). Both are equally well-suited for moderate heme binding based on the HXXY motif and a positive net charge. Within the scavenger receptor cysteine-rich domain of the enzyme (Mendes de Oliveira et al., 2018), the interesting motif KKL**YH**^{227}SDAC was found. It features an YH motif of intermediate heme-binding affinity on peptide level, however, of markedly improved affinity on the protein level as earlier demonstrated for IL-36α (Wißbrock et al., 2019; Syllwasschy et al., 2020). Furthermore, it shows high net charge and a hydrophobic leucine, which likely leads to high heme-binding affinity. A comparative analysis of all motifs revealed that the S protein was the only SARS-CoV-2-derived protein with a promising HBM (FLGV**Y**^{144}**YH**KN). In contrast, the human proteins ACE2 and TMPRSS2 showed both quantitatively and qualitatively superior motifs. In ACE2, LTA**HH**^{374}EMG**H** and PL**Y**E**H**^{239}L**H**A**Y** and SFIR**Y**^{515}**Y**TRT boast promising H/Y motifs and

favorable properties. Likewise, TMPRSS2 contains e.g. the motif KVIS**H**^{334}PN**Y**D, which represents a potential heme-interaction sites directly in the catalytic protease domain, and KKL**YH**^{227}SDAC, which might also bind heme efficiently.

3.2 Heme binding to protein 7a- and ACE2-derived peptides

The predicted motifs for protein 7a (2 motifs) and ACE2 (5 motifs) were synthesized as nonapeptides (Table S1) in order to support our findings with experimental data. While motif DGVK**H**^{73}V**Y**QL didn't bind heme at all, heme binding was observed for the second motif (VK**H**V**Y**^{75}QLRA). These motifs are only shifted by two amino acids, and thus overlaying, but based on the N-terminal asparagine acid residue, the first motif (DGVK**H**^{73}V**Y**QL) has a more acidic character which abolishes binding of heme. Unfortunately, for the second motif (VK**H**V**Y**^{75}QLRA) no binding affinity could be determined (n. sat.), but binding was observed by a shift of heme's Soret band to ~419 nm (Figure 2A).

In line with the predictions, ACE2 has more heme-binding motifs with higher binding affinity (Figure 2B). Indeed, heme did not bind to the peptide with the motif LTA**HH**^{374}EMG**H**, which might be due to the glutamic acid residues. Furthermore, QAAK**H**^{535}EGPL did not bind heme. In contrast, all other ACE2-derived peptides bound heme with moderate or high affinity. Thereby, SFIR**Y**^{515}**Y**TRT bound heme most efficiently with a K_D of 0.60 ± 0.33 μM (λ ~424 nm; Figure 2B) and, thus, is the most promising heme-binding motif of ACE2. The peptides with the motifs PL**Y**E**H**^{239}L**H**A**Y** and AMRQ**Y**^{654}FLKV showed moderate binding of heme with a K_D of 4.04 ± 1.20 μM (λ ~415 nm) and 5.01 ± 0.78 μM (λ ~432 nm), respectively (Figure 2B).

3.3 Pathophysiological effects of heme and COVID-19 intersect at inflammation

In order to shed light on the crosstalk and common pathways between heme and COVID-19, we investigated the overlap between our two KGs (i.e., heme KG (Humayun et al., 2020) and the COVID-19 KG (Domingo-Fernández et al., 2020)) (Figure 3). While the Heme KG was generated from the analysis of 46 scientific articles specifically selected to explain inflammatory processes related to labile heme, the COVID-19 KG contains over 150 articles. The difference in the size of these KGs thus explains the disproportionate number of molecules they possess (Figure 4A). Nonetheless, we observed that a significant amount of proteins is shared, predominantly in three major systems, namely blood coagulation, complement and immune system. Among these 85 shared nodes, there are 45 clinical phenotypes, 35 proteins, 4 immune system specific cells, and 5 small molecules. 27 nodes belong to immune response evoking (pro-)inflammatory pathways, 4 to the complement system, and 22 to the blood coagulation system (Figure 4A). Moreover, we also noticed the presence of 7 clinical phenotypes related to organ dysfunction. Further, we individually investigated the four systems to reveal the common relations observed in each of the two KGs (Figure 4, Table S2-S5). Finally, we compared the directionality of these relations (i.e., up-/down-regulation) against experimental data published in the context of COVID-19 (Blanco-Melo et al., 2020). We found that the vast majority of the observed dysregulations were concordant with our findings (Table S6).

The largest consistency was found in inflammatory pathways (Figure 4B) as indicated by a common set of inflammatory – mostly pro-inflammatory – molecules. These molecules are changed with respect to their levels due to expression and/or secretion or their activity as a consequence of both, high heme concentrations and COVID-19 infection, mediating inflammatory response. In particular, the pro-inflammatory cytokines TNFα, IL-1β, IL-6, IL-8, and the anti-inflammatory cytokine IL-10, as well as proteins related to TLR4-mediated signaling pathways (i.e. CD14, MyD88, NF-κB and TLR4) are influenced under both conditions (Figure 4B, Table S2).

Within the complement system (Figure 4C, Table S3), one of the main mediators, C3, is activated under hemolytic conditions, which are associated with high heme concentrations, thus leading to complement activation (Roumenina et al., 2016). The same was observed in COVID-19 patients (Risitano et al., 2020). Furthermore, other complement factors, including C5a and C1q, were reported to be activated by heme (Roumenina et al., 2016). So far, an increase of the activation of these proteins was not described for COVID-19. Finally, the number of neutrophils is positively correlated with both heme and COVID-19 infection. However, heme induces neutrophil activation through a ROS-dependent mechanism (Dutra and Bozza, 2014), a pathway that is not yet discussed in the context of COVID-19.

The blood and coagulation system is pronounced by the connecting proteins ferritin and albumin (Figure 4D, Table S4). Both conditions lead to reduced levels of ferritin, a protein involved in iron uptake and release (Mogl, 2007). Same applies for albumin in COVID-19 patients (Chen et al., 2020; Guan et al., 2020; Yang et al., 2020; Zhou et al., 2020a). Moreover, albumin is known as one of the common heme scavengers, neutralizing heme's toxic effects up to a certain extent (Roumenina et al., 2016). As indicated by the impact on different components of the blood coagulation system, such as plasminogen or fibrin in case of COVID-19 and heme, respectively, both conditions can influence hemostasis. With regard to the impact on platelets, a decreased platelet count was observed in COVID-19 patients (Wang et al., 2020), whereas for heme an induction of platelet aggregation was described (Roumenina et al., 2016).

Finally, a trend towards elevated levels of organ-specific diagnostic markers, i.e. LDH and bilirubin, is shared by both KGs (Figure 4E, Table S5).

4 Discussion

Currently, SARS-CoV-2 and its associated disease COVID-19 keep the world in suspense. Patients being most severely affected suffer from pneumonia, acute respiratory distress syndrome, and death (Ragab et al., 2020; Ye et al., 2020; Zhou et al., 2020a). While COVID-19 patients often exhibit high levels of proinflammatory markers as well as an activation of the complement and coagulation system, hemoglobin and albumin levels have been reported to be remarkably low (Chen et al., 2020; Risitano et al., 2020; Whetton et al., 2020; Ye et al., 2020). In contrast, there is evidence for an occurrence of higher level of hemopexin and haptoglobin in COVID-19 patients (Whetton et al., 2020). These affected clinical parameters have generated a recent debate about the role of heme in the context of COVID-19 that has not been conclusively explained to date (Cavezzi et al., 2020; Comentale et al., 2020; Liu and Li, 2020; Wagener et al., 2020).

With this work, we intend to provide deeper insights into the potential of a correlation between SARS-CoV-2 infection, COVID-19 and the effects of heme, wherever possible and appropriate. Such a connection would be in line with recent studies that already described the impact of heme in the context of different viruses (Lecerf et al., 2015; Gupta et al., 2015; Neris et al., 2018). Lecerf et al. reported on the interaction of heme with antibodies (Abs) resulting in the induction of new antigen binding specificity and acquisition of binding polyreactivity to gp120 HIV-1 in 24% of the antibodies from different B-cell subpopulations of seronegative individuals (Lecerf et al., 2015). In contrast, no difference in the sensitivity towards heme was found for Abs originally expressed by naive, memory, or plasma cells. The transient interaction of heme with a fraction of circulating Abs that might change their antigen binding repertoire by means of cofactor association was suggested as another possible regulatory function of heme (Lecerf et al., 2015). In addition, the novel antigen specificities of these circulating Abs was proposed to be recruited only in case of certain pathological conditions that might depend on extracellular heme as occurring in disorders such as malaria, sickle cell disease, hemolytic

anemia, β-thalassemia, sepsis, and ischemia-reperfusion (Lecerf et al., 2015). A similar report by Gupta et al. revealed the heme-mediated induction of monoclonal immunoglobulin G1 antibodies that acquired high-affinity reactivity towards antigen domain III of the Japanese encephalitis virus (JEV) E glycoprotein that exhibited neutralizing activity against dominant JEV genotypes (Gupta et al., 2015). In both cases, heme was found to confer novel binding specificities to the respective Abs without changing the binding to their cognate antigen and, as a consequence of the contact with heme, the anti-inflammatory potential of these Abs was substantially increased (Gupta et al., 2015). Finally, Assuncao-Miranda et al. and Neris et al. described the inactivation of different arthropod-borne viruses like Dengue, Yellow Fever, Zika, Chikungunya, Mayaro and others by porphyrin treatment via targeting of the viral envelope and thus, the early steps of viral infection (Assunção-Miranda et al., 2016, Neris et al., 2018). All together, these studies advocate for studying the impact of heme in coronavirus-infected patients (Figure 5).

Here, we have investigated the possibility of a direct interaction of heme with SARS-CoV-2 surface proteins and their human counterparts ACE2 and TMPRSS2. Our analysis revealed that heme binding conferred by HBMs would potentially be possible. The quality, availability, and accessibility of the motifs follows the rank order: TMPRSS2 (good binder) > ACE2 > S protein > Protein 7a (poor binder). Especially in TMPRSS2, the location of the most suitable HBMs correlates with the important catalytic protease domain. This potential heme interaction would be of a transient nature, as has been observed for other heme-binding proteins such as IL-36α, APC and CBS (Kumar et al., 2018; Wißbrock et al., 2019; Hopp et al., 2020). Intact heme would bind to the protein surface in a reversible fashion, which would be in contrast to the recently presented hypothesis by Liu & Li (Liu and Li, 2020). Therein, the authors describe heme extraction from hemoglobin through attack by viral proteins and subsequent iron and porphyrin release from heme, which does not occur in a physiological situation (Belcher et al., 2010). In addition, the docking analysis performed in their study is not based on experimental data concerning the porphyrin interaction, unlike the data-driven machine learning algorithm HeMoQuest (Paul George et al., 2020) used in our study. Nevertheless, the effect of heme on the suggested proteins TMPRSS2, ACE2, S protein, and Protein 7a needs experimental verification. Thus, in order to further prove these assumptions, UV/vis heme binding studies with protein 7a- (as a virus protein representative) and ACE2 (as a human host cell protein representative) were conducted. Indeed, for protein 7a the central heme-binding motif VK**H**V**Y**^{75}QLRA was confirmed (Figure 2A). From the pool of potential heme-binding motifs, that were predicted for ACE2, three motifs turned out as suitable motifs, with moderate to high heme-binding affinity (Figure 2B). Within the past, heme-binding studies on the level of protein-derived peptides as a tool for the evaluation of motifs on protein level was successful to characterize heme-binding to several proteins, among them IL-36a and APC (Wißbrock et al., 2019; Hopp et al., 2020). However, binding and functional studies on the protein level for COVID-19-derived proteins are still required to enable a complete assessment of the suggested interactions.

Apart from investigating the direct impact of heme on proteins at the interface of the virus-host cell interaction, we also explored similarities between relevant pathways characterizing the respective pathologies, i.e. labile heme occurrence in hemolytic conditions and COVID-19 disease progression (Figure 5). Interestingly, in the literature we found several intersections: Both, hemolytic conditions and COVID-19, have been found to trigger inflammatory pathways. COVID-19 patients often develop respiratory distress syndrome, which is accompanied by a cytokine storm, and thus an activation of the immune system (Ye et al., 2020). Clinically, this is manifested by an increase in the levels of a wide range of cytokines, including TNFα, IL-1β, IL-6 and IL-8 (Ye et al., 2020), and the activation of the complement system (e.g. C3) (Risitano et al., 2020; Skendros et al., 2020). Interestingly, hemoglobin is described to be often decreased in COVID-19 patients (Huang et al., 2020; Whetton et al., 2020). The underlying molecular basis of this phenomena is not yet identified and so far there is no evidence

for a change of hemoglobin's oxygen binding affinity in COVID-19 patients as earlier suggested by Liu and Li (Daniel et al., 2020; DeMartino et al., 2020; Huang et al., 2020; Liu and Li, 2020). However, the lower levels of hemoglobin seem to correlate with increased levels of the iron-storage protein ferritin. Such an increase in ferritin is observed in diseases like hemochromatosis or porphyria (Mogl, 2007). Furthermore, it is upregulated during hemolytic diseases as a consequence of hemoglobin degradation and the associated increase of oxidative stress, e.g. induced by heme (Belcher et al., 2010). Hemolytic disorders such as malaria, ischemia-reperfusion, hemorrhage or hemolytic anemias are associated with an excess of labile heme and are, as in COVID-19 infection, often accompanied by inflammatory events (Chiabrando et al., 2014; Barcellini and Fattizzo, 2015). Therefore, similar clinical parameters are observed under these conditions (Barcellini and Fattizzo, 2015). Moreover, several studies have reported that heme directly binds or induces TNFα, IL-1β and IL-8, and triggers numerous inflammatory pathways (e.g. NF-κB signaling) (Dutra and Bozza, 2014; Humayun et al., 2020). Taken together, these clinical observations suggested a correlation between both processes, which we aimed to analyze by superimposing the two KGs of both pathophysiologies (Domingo-Fernández et al., 2020; Humayun et al., 2020). We integrated Heme KG with COVID-19 KG to identify these similarities, like we have done successfully, for example, for Type II Diabetes and Alzheimer's disease (Kodamullil et al., 2015; Karki et al., 2017; Karki et al., 2020). Indeed, the results of the knowledge-driven analysis revealed a core of similar shared molecular patterns. The majority of these were related to the three major systems inflammation, complement, and coagulation system. As expected, inflammation was the most emphasized and shared system, suggesting several processes that are commonly mediated by both heme and in COVID-19. The TLR4 signaling pathway was previously shown to play an important role in heme-mediated inflammatory processes. Interestingly, this pathway with its components TLR4, MyD88 and NF-κB was pronounced in the overlay of the heme KG and COVID-19 KG. The TLR4 pathway belongs to the innate immune system, and thus results in the production of several proinflammatory cytokines, such as TNFα, IL-1β and IL-6 (Humayun et al., 2020). TNFα and IL-1β can further stimulate the release of inflammatory mediators, such as IL-8. Exactly the same proteins have emerged as common key molecules in our analysis. Clinical observations revealed their upregulation in COVID-19 patients as well as during hemolytic events (Dutra and Bozza, 2014; Humayun et al., 2020; Ye et al., 2020), which highlights even more the TLR4 signaling pathway in both situations. Interestingly, TNFα and IL-1β were reported to be capable of regulating platelet aggregation. This might support the common link of both pathologies to blood coagulation (Bar et al., 1997). Blood parameters, such as hemoglobin and albumin levels, may allow for a direct correlation between COVID-19 and heme, since they are inevitably connected to the processing of heme under hemolytic conditions (Chiabrando et al., 2014; Roumenina et al., 2016). At the current state of research, there is no explanation for the decreased levels of hemoglobin in COVID-19. It might be conceivable that this is due to a rapid turnover of red blood cells, which would lead to a degradation of hemoglobin and, in turn, to an increase of heme. As for other viruses (e.g., hepatitis A (Goel et al., 2018)), just recently, SARS-CoV-2 infections were associated with anemia, hemolytic and/or hemorrhagic conditions (Capes et al., 2020; Conti et al., 2020; Lazarian et al., 2020; Sahu et al., 2020; Vishnu et al., 2020), which further supports the importance of the results gained within this study with respect to a need of development of tailor-made diagnosis and therapy for these patients.

However, our approach is not without limitations as our analysis is restricted to a limited number of scientific articles. Furthermore, there is an unbalanced source of information when comparing the tremendous amount of literature that is currently being published on COVID-19 versus the information that is currently included in heme KG for heme. The findings described herein require a more detailed experimental investigation with dedicated experiments for each of the reported relations that shed light on the underlying biochemical mechanisms as well as for the full characterization of the heme-binding capacity of the proposed proteins. Nevertheless, the results of this study draw attention to a relationship

that could be plausible based on the current characterization of COVID-19 by clinical parameters. A correlation between the symptoms of COVID-19 infection and the consequences of excess heme does not necessarily have to be related, but in specific cases it may correlate or even cause a more severe course of the disease in pre-existing hemolytic conditions or hemolysis-provoking events.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

M.H.A. and D.I. designed and planned the project. M.-T.H., D.D.-F., Y.G., M.S.D, B.F.S. and R.G. performed the experiments and collected the data. Data analysis was carried out by all authors. The manuscript was written through the contribution of all authors.

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Figure legends

Fig. 1: Potential heme-binding proteins on the virus and host cell surface. Four COVID-19-related proteins, namely the virus proteins (grey) **(A)** S protein and **(B)** protein 7a as well as the host cell proteins (yellow) **(C)** ACE2 and **(D)** TMPRSS2, turned out as possible heme-binding proteins. The location of the proteins is presented (first column, left), individually highlighting each target protein (S protein, red; protein 7a, orange; ACE, green; TMPRSS2, turquoise). All motifs predicted by HeMoQuest (Paul George et al., 2020) are shown excluding those with modifications (glycosylation, disulfide bonds) or located in intracellular or virion domains, i.e. 24 motifs for S protein, 2 motifs for protein 7a, 15 motifs for ACE2, and 14 motifs for TMPRSS2. Potential heme-binding residues are bold-written and numbered according to the SwissProt numbering system (Bairoch and Apweiler, 2002). A refined analysis considering the surface accessibility of the motifs resulted in 3 motifs for S protein, 2 largely overlapping motifs for protein 7a, 5 motifs for ACE2, and 10 motifs for TMPRSS2 (third column). In addition, these motifs are highlighted in a zoom-in below the list with annotation of the respective potential coordinating residues (green; third column), as well as in the available monomer (fourth column) and oligomer (fifth column) structures, if applicable (S protein, homology model from C-I-TASSER (Zhang et al., 2020a); protein 7a, in-house homology model; ACE2, PDB: 6M18; TMPRSS2, Swiss-model: O15393). Within the oligomers, the motifs were only depicted in one of the monomers (green). Each time, the central, potential heme-coordinating residue is shown as a stick model. Since some surface-exposed motifs within S protein were not covered by the available EM structure (PDB: 6VXX), motifs were highlighted within the monomer homology model from C-I-TASSER (Zhang et al., 2020a) (turquoise), which was then superimposed with the trimer (PDB: 6VXX). Where applicable, glycosylation sites and ions are highlighted in blue.

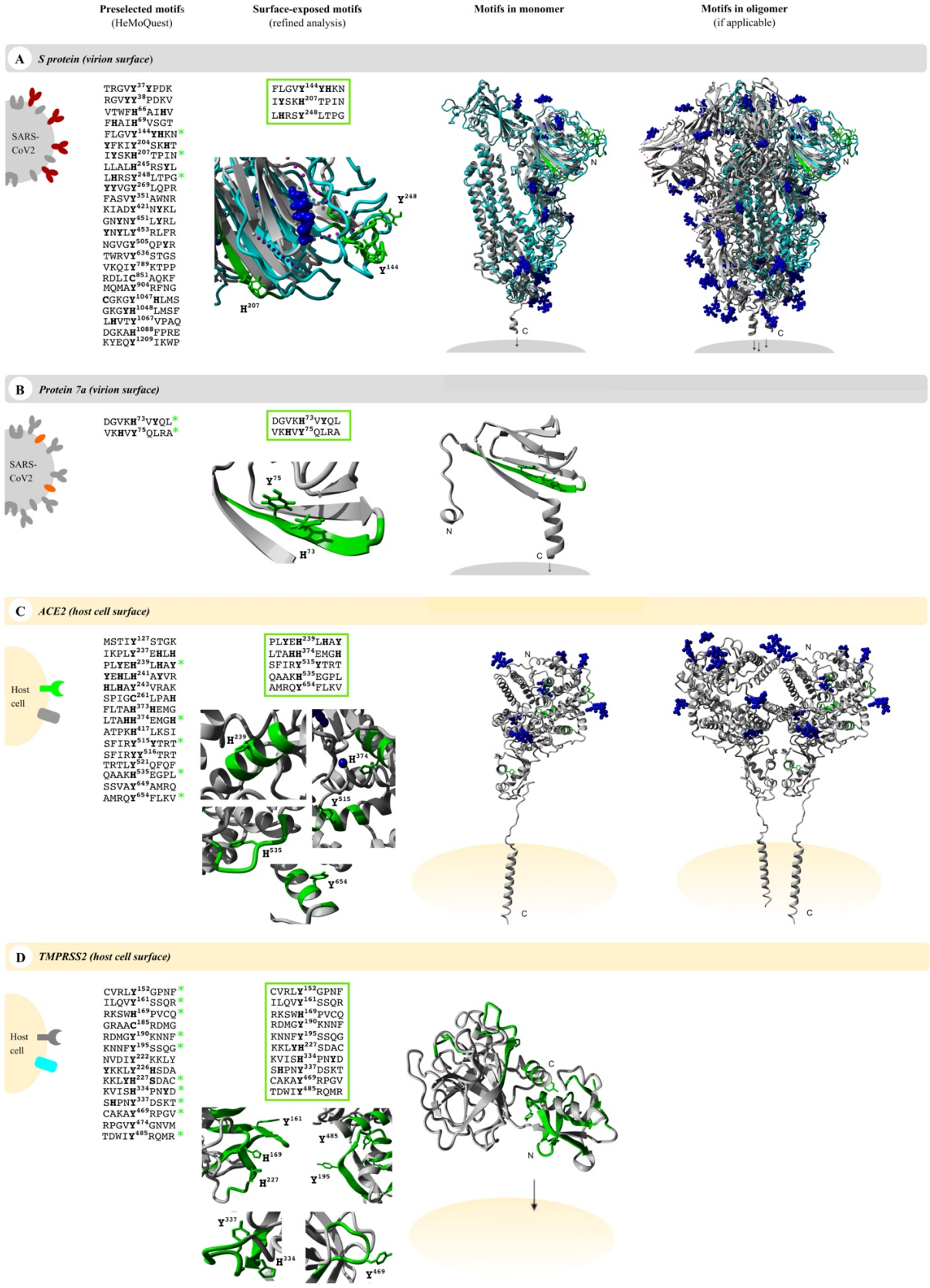
Fig. 2: Heme-binding capacity of potential HBMs in protein 7a and ACE2. Heme (0.4 - 40 µM)-binding properties of the motif-derived peptides (10 µM) were investigated by UV/vis spectroscopy. The locations of the motifs within the proteins' structures (left) and the difference spectra (right) are depicted. **(A)** For SARS-CoV-2 protein 7a, VK**H**V**Y**^{75}QLRA was confirmed as a heme-binding motif. A shift of the Soret band to ~419 nm was observed (right). However, the determination of a K_D was not possible. Within the small protein (extravirion domain shown) the motif (yellow) is found within a β-sheet, suitably exposed for a potential interaction with heme. **(B)** Within human ACE2, three heme-binding motifs were confirmed. All of them induced a Soret band shift to ~415 - ~532 nm in complex with heme (right). SFIR**Y**^{515}**Y**TRT (green) exhibit the highest heme-binding affinity (K_D = 0.60 ± 0.33 µM; right), suggesting it as a highly probably heme-binding site within ACE2. Moreover, two further motifs, PL**Y**E**H**^{239}L**H**A**Y** and AMRQ**Y**^{654}FLKV, were characterized as moderate heme-binders (yellow) with a K_D of 4.04 ± 1.20 µM and 5.01 ± 0.78 µM, respectively. Therefore, there might be even two more heme-binding sites at the surface of ACE2. SFIR**Y**^{515}**Y**TRT and PL**Y**E**H**^{239}L**H**A**Y** are found within the metallopeptidase domain of ACE2, whereas AMRQ**Y**^{654}FLKV is part of a region that is essential for cleavage by the ADAM17.

Fig. 3: Overlap between the COVID-19 KG (Domingo-Fernández et al., 2020) and Heme KG (Humayun et al., 2020). (A) The network displays the largest area of overlap of non-molecular interactions between the two KGs. Node coloring denotes whether a particular entity is present exclusively in the COVID-19 KG (blue), in the Heme KG (green), or in both (red). Finally, neighbors of overlapping nodes are colored in light red. The parts of the network which correspond to three major systems (i.e. inflammation, blood coagulation and complement system) have been circled. **(B)** High resolution network view of the inflammation system. Here, directionality of the relations can be seen. Solid edges represent increase and decrease relations while dashed edges represent correlations.

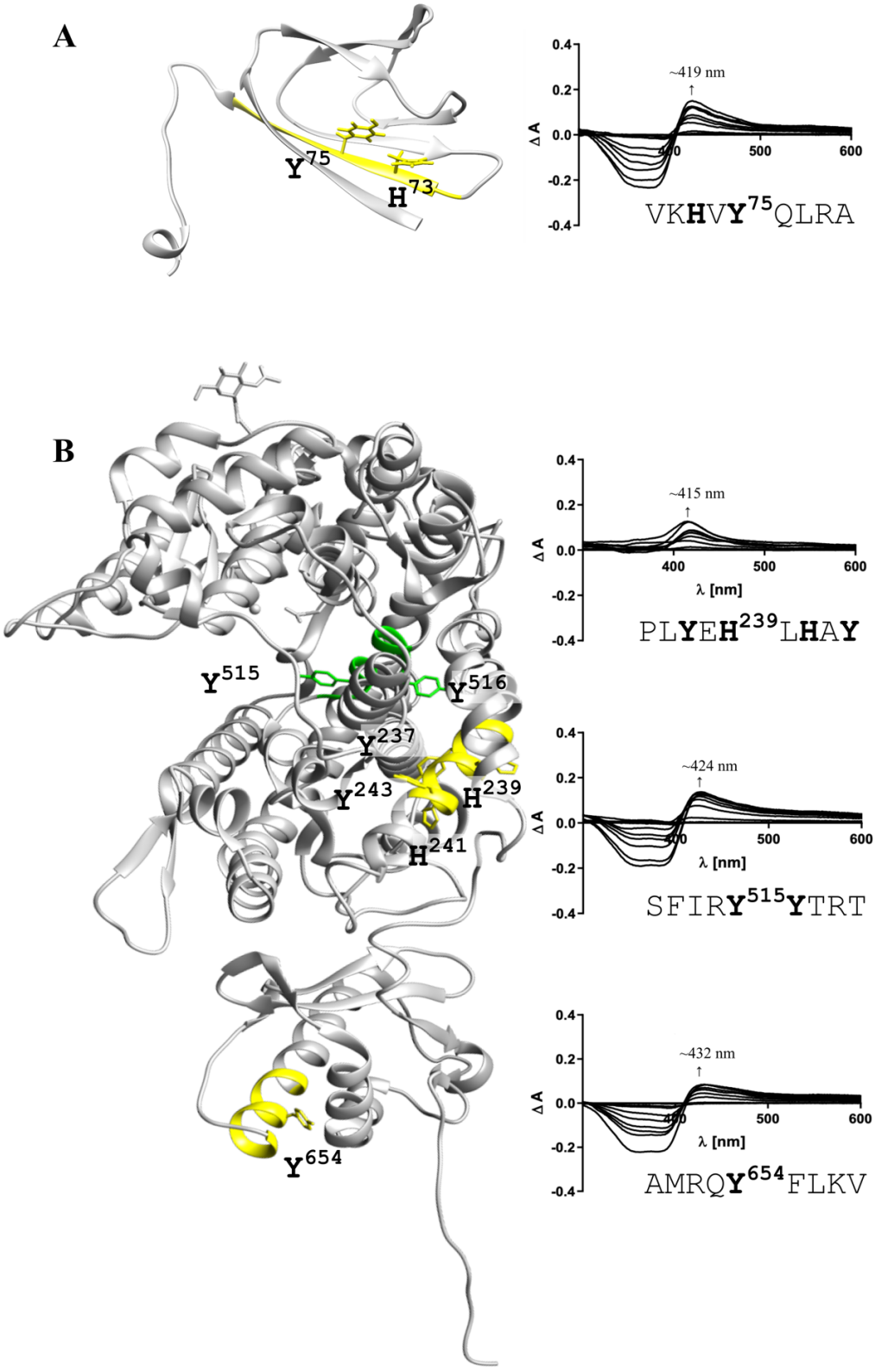
Fig. 4: Shared biochemical pathways based on the overlap between COVID-19 KG (Domingo-Fernández et al., 2020) and Heme KG (Humayun et al., 2020). (A) Overlap between the two KGs based on human proteins represented as a Venn Diagram. The numbers of nodes that only present proteins are depicted (595 nodes in COVID-19 KG, 106 nodes in Heme KG and 32 nodes as an overlap between both KGs). In particular, the two KGs overlap in the following systems: **(B)** Immune response - inflammation, **(C)** immune response - complement system, **(D)** blood and coagulation system, and **(E)** organ-specific diagnostic markers. Moreover, for each classification available clinical parameters were denoted (Chen et al., 2020; Risitano et al., 2020). CRP = C-reactive protein, C3 = Complement component 3, CD = Cluster of differentiation, G-CSF = Granulocyte-colony stimulating factor, GM-CSF = Granulocyte macrophage colony-stimulating factor, IL = Interleukin, LDH = Lactate dehydrogenase, MCP1 = Monocyte chemoattractant protein 1.

Fig. 5: COVID-19 infection and hemolysis show common changes of clinical parameters. Top left: Virus (grey) release after conquering the host cell (yellow) and taking over its protein synthesis machinery. Top right: In case of hemolysis, erythrocyte lysis occurs in a blood vessel, leading to degradation of hemoglobin (blue/red, PDB: 1GZX) and, thus, to an excess of labile heme. Circulating erythrocytes (red) and platelets (bright blue) are shown. Bottom left: Interaction of virus and host cell before cell entry. S protein (light red, PDB: 6VXX) on the surface of SARS-CoV-2 (grey), interacts with human ACE2 receptor (green, PDB: 6M18). The protease TMPRSS2 (turquoise) primes S protein and contributes to cell entry (Hoffmann et al., 2020). The accessory protein 7a (in-house homology model) interacts with S protein, M protein and E protein for virus particle assembly. E protein is discussed to form ion channels and to play a role in viral genome assembly (Ruch and Machamer, 2012). M protein may be relevant for entry and attachment of the virus as well as for the budding process (Bianchi et al., 2020). The shown proteins contain domains in the extracellular space that have specific characteristics for heme binding. Bottom right: Prominent changes of clinical parameters in patients suffering from COVID-19 infection (↑ increase, ↓ decrease). The terms depicted by an asterisk, have been reported in both, hemolysis and COVID-19 infection (Barcellini and Fattizzo, 2015; Chen et al., 2020).

Hopp et al., Figure 1



Hopp et al., Figure 2



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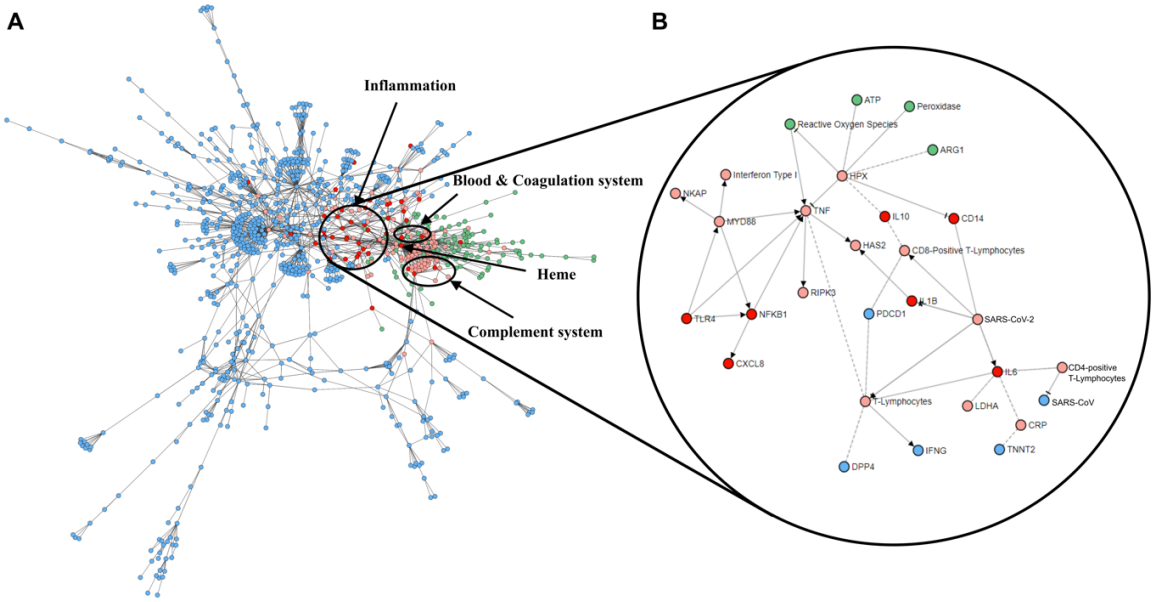

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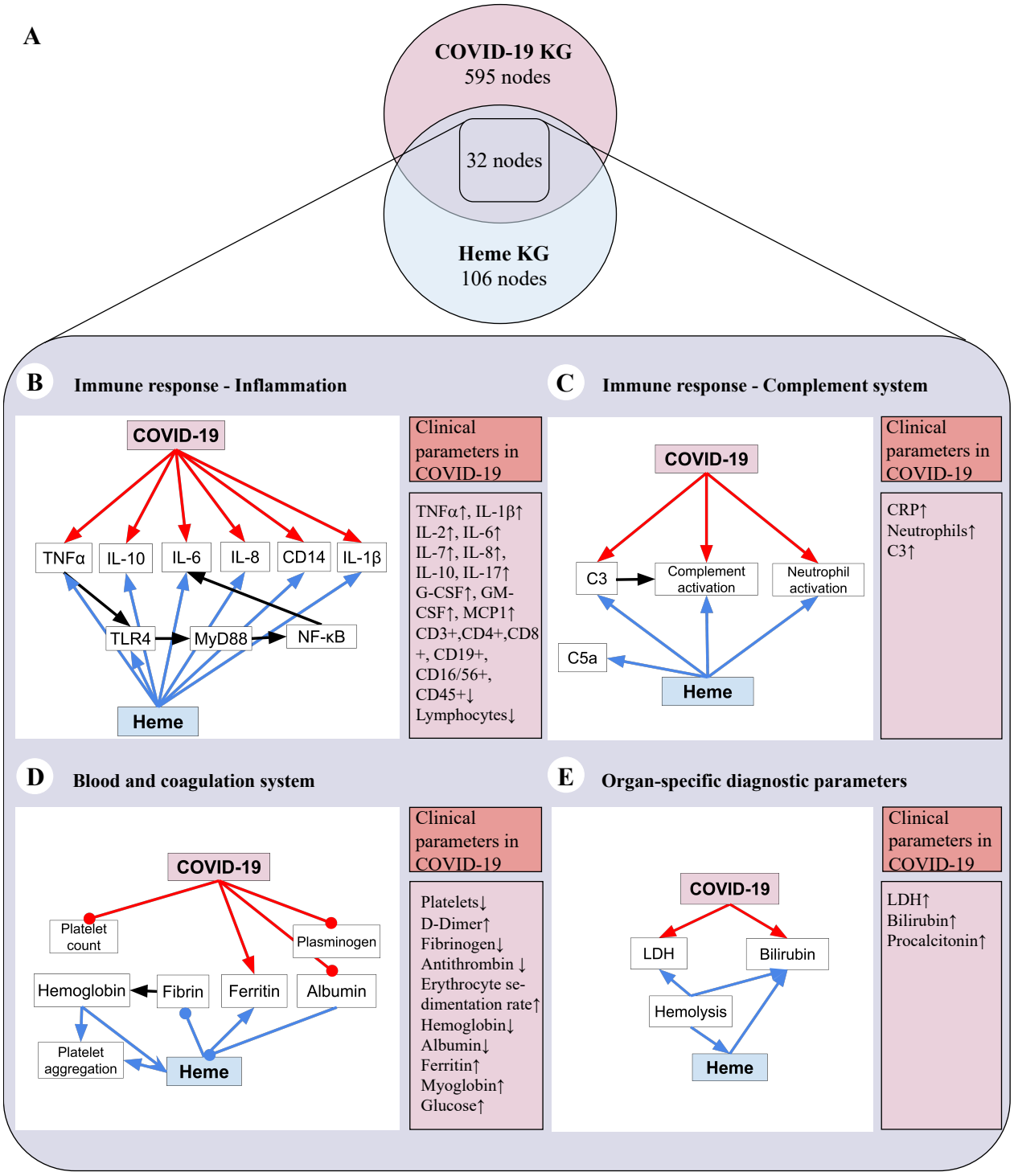
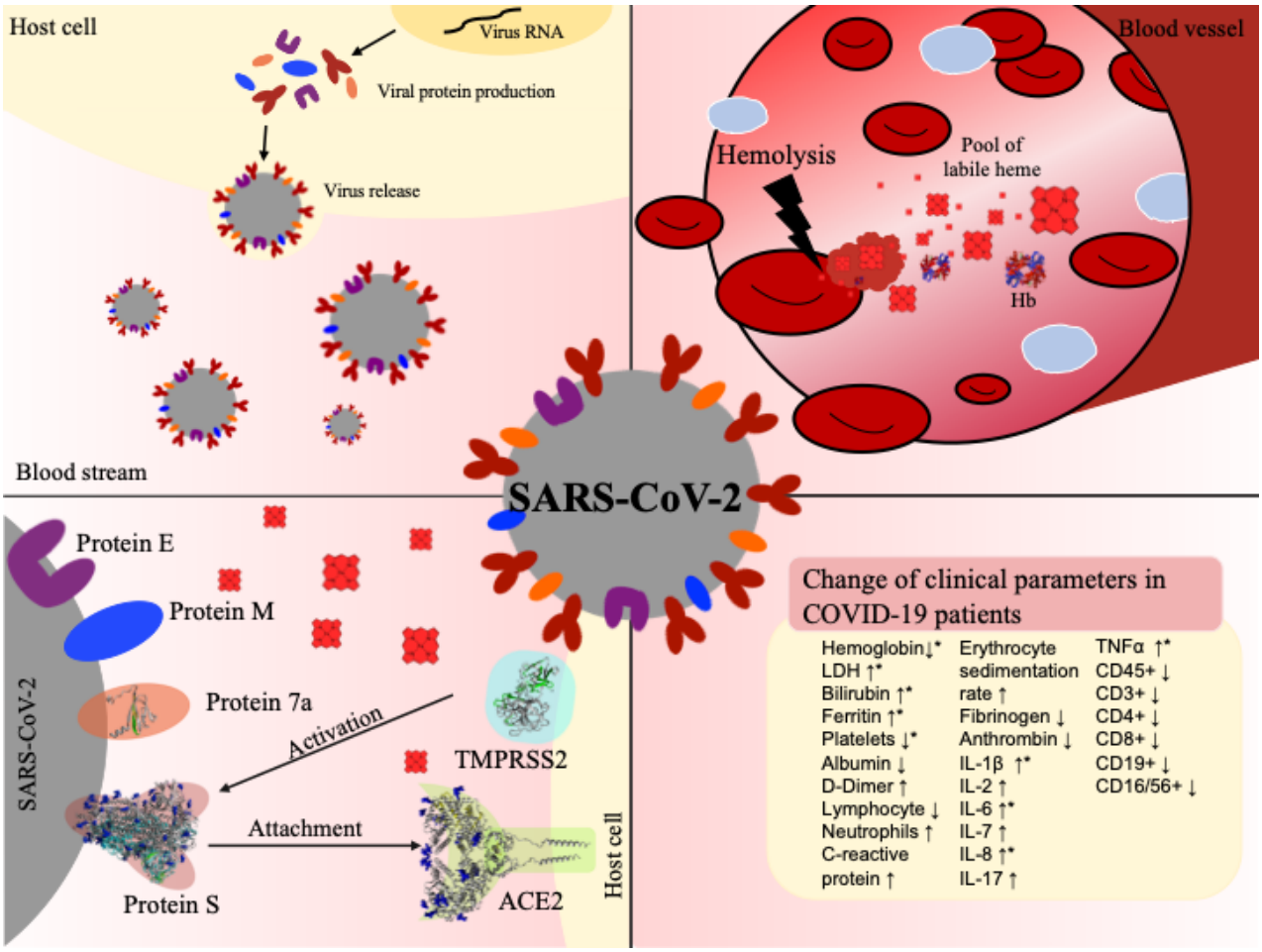


Hopp et al., Figure 5



4.4.2 Summary

Extracellularly available proteins that participate in the entry of SARS-CoV-2 into host cells were analyzed for potential HRMs by applying HeMoQuest.[431] Thereby, two virus (i.e., spike glycoprotein and protein 7a) and two host (i.e., angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2)) cell proteins were suggested as potential heme-binding proteins. Subsequent manual exclusion of motifs with disulfide and glycosylation sites or location beyond the extracellular domain revealed two and three potential HRMs in the extravirion domain of protein 7a and spike glycoprotein, respectively. In comparison, the human proteins ACE2 and TMPRSS2 possess potential HRMs of higher quantity (five (ACE2) and 10 (TMPRSS2)) and quality. As exemplified with one virus-derived (protein 7a) and one host cell-derived (ACE2) protein, these predictions were experimentally explored. HRMs were synthesized as protein-derived nonapeptides and studied for their heme-binding capacity. While binding to one protein 7a-derived peptide was shown, three of the ACE2-derived peptides bound heme with moderate to high affinity, justifying future studies on the protein level.

Apart from the prediction of potential heme-binding proteins of the virus and host cells, pathological effects of hemolysis and COVID-19 were compared through the overlay of two separate knowledge graphs, HemeKG (cf. Chapter 4.3) and COVID-19 KG[453]. Both pathologies overlapped in the increase of several biomarkers related to inflammation (e.g., TNFα, IL-8 and IL-1β), complement system (e.g., C3), blood and coagulation system (e.g., ferritin) and organ specific diagnostic parameters (e.g., LDH).

Hence, this publication suggests a potential correlation of heme and COVID-19 pathologies, especially with respect to inflammation, complement and coagulation activation. Thus, preexisting hemolytic disorders might even potentiate COVID-19 symptoms through either parallel induction of inflammation and coagulation or a direct impact of heme on the virus entry by interaction with relevant proteins. To what extent hemolytic events play a role in the COVID-19 pathology itself is not known to date.

5 Conclusions

Hemolysis-derived labile heme has been associated with a variety of toxic effects, comprising the promotion of proinflammatory pathways as well as the activation of the complement and the coagulation system.[6–11] As a consequence, patients with hemolytic disorders suffer in particular from systemic inflammation and thrombotic complications, such as DVT.[12–17] However, the molecular basis of these heme-triggered symptoms is not yet entirely understood. The present thesis is devoted to the role of heme as a procoagulant alarmin with the aim to promote the research on hemolysis-driven thrombosis.

First of all, evidence for heme-driven disturbance in the coagulation system as well as reports on interactions of heme with components of the same were collected and contextualized, displaying the versatile impact of heme on the cellular and the molecular level (cf. Chapter 4.1, Figure 8, Figure 9). Initially, heme activates the endothelium, which is mediated through the induction of several signaling pathways (Figure 8a, Figure 9). Thereby, morphological changes, loss of barrier integrity, cell detachment, and denudation are consequences of heme exposure.[196,203,204,390–394] Heme-stimulated TLR4 signaling leads to increased adhesion protein expression, WPB exocytosis, and collagen exposure, which further supports prothrombotic reactions (Figure 8, Figure 9).[197,206–210,397] While heme activates platelets through direct binding to CLEC2, aggregation is for example promoted by heme-triggered α-granules exocytosis.[18,400,402] Heme-driven TLR4 signaling induces leukocyte rolling and adhesion to the endothelium as well as increased TF expression (Figure 8a, Figure 9).[210,213,407–411]

For all of these heme-driven cellular processes the temporal and local sequel as well as the complete signaling chronology has not yet been characterized, but one pathway is highly pronounced, i.e. the TLR4 signaling pathway (Figure 9). Interestingly, the same pathway emerged in the independent knowledge-based computational analysis performed in this thesis (cf. Chapter 4.3). Data curation from 46 heme-related articles resulted in HemeKG, a knowledge graph comprising the effects of heme under hemolytic conditions. Although only information from a first selection of articles has been analyzed, effector molecules of the TLR4 signaling pathway were particularly dominant. After enrichment with data from pathway databases and further literature screening, specific effector molecules were identified that were not yet described in the context of heme-driven TLR4

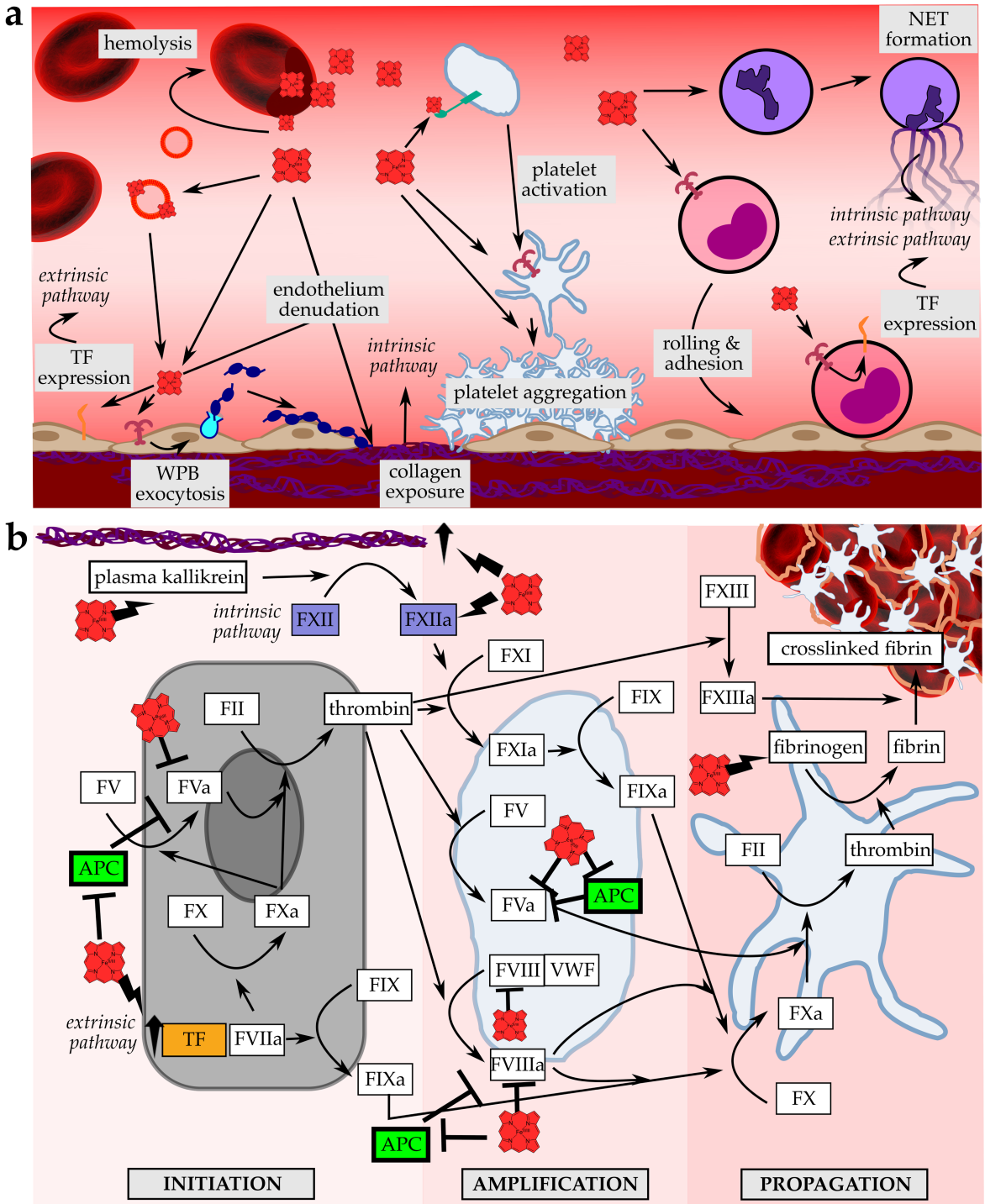


Figure 8: Schematic overview of the current knowledge of heme's procoagulant function. Heme affects **a)** cellular components **b)** and proteins involved in the process of blood coagulation. APC (green) is depicted as the only inhibitor, as it has been identified in the present thesis as a so far unknown heme-regulated protein. Signaling pathway information on the cellular level are separately depicted in Figure 9.

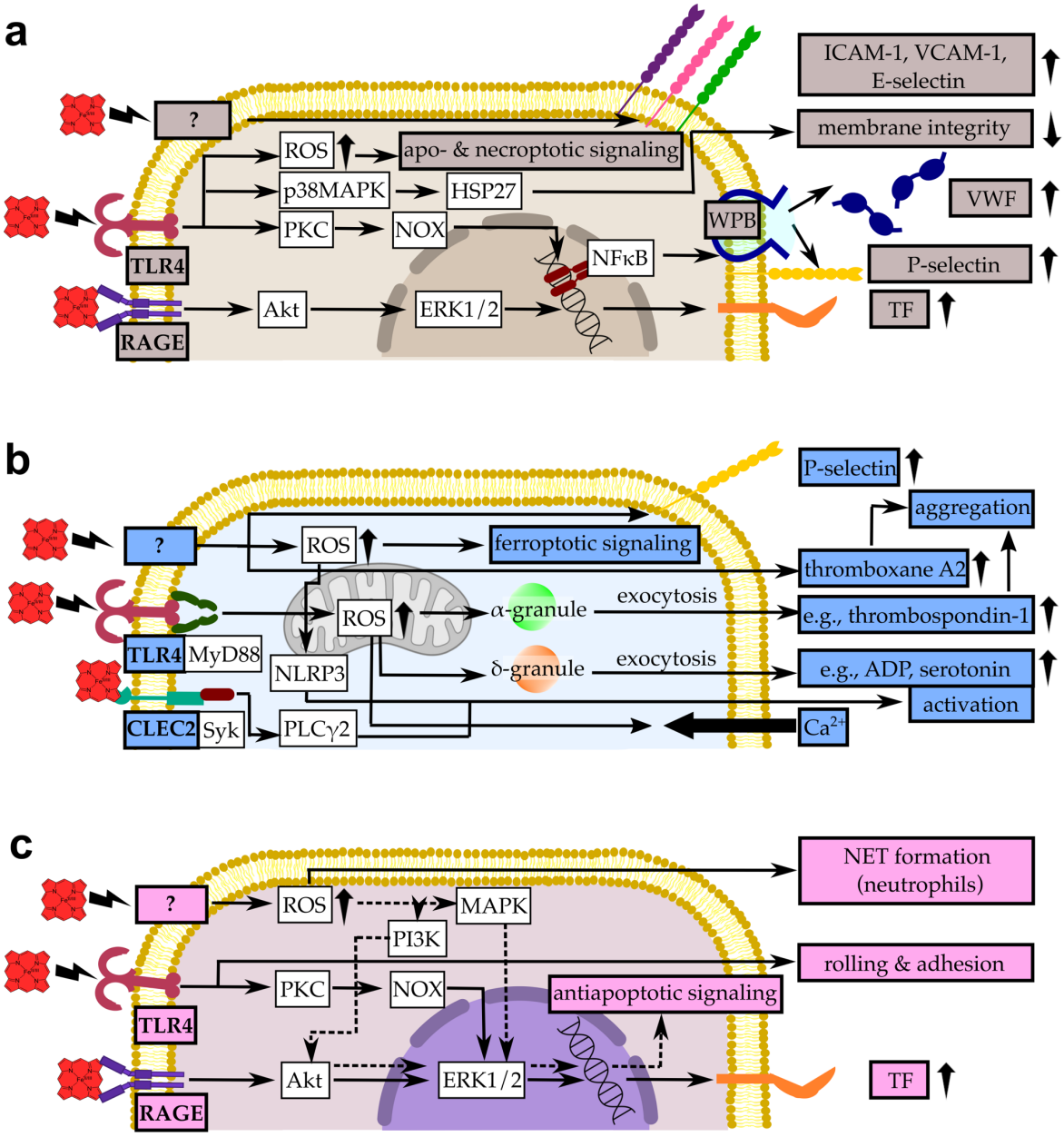


Figure 9: Overview of the signaling pathways triggered by heme in the context of hemostasis. Many processes in **a)** endothelial cells, **b)** platelets, and **c)** leukocytes are initiated by either TLR4 or ROS generation. **a)** Heme-triggered TLR4 signaling results in apoptosis and necroptosis in endothelial cells, as well as a loss of membrane integrity and WPB exocytosis.[196,197,203,204] **b)** Exocytosis of α-granules by platelets results from TLR4 activation by heme as well.[400,402] **c)** Within leukocytes, heme-driven TLR4 signaling causes rolling and adhesion to the endothelium as well as the upregulation of TF expression.[210,213,407–411] Only receptors and effector molecules are depicted that emerged from the literature search within the scope of heme-driven procoagulant reactions (cf. Chapter 4.1). Further investigations are required to explore the interrelations of the shown signaling pathways in their entirety.

signaling (e.g., TIRAP and IRAKs), and may thus represent suitable targets for future research. However, the selection of articles was limited in this study and mainly referred to the proinflammatory role of heme, linking TLR4 activation by heme and the increasing cytokine levels (e.g., TNFα and IL-1β) under hemolytic conditions, but not with the procoagulant effects of heme. The therein presented TLR4 signaling pathway does not fit well to the suggested TLR4 signaling in the context of heme-driven coagulation and needs further exploration (Figure 9). The inclusion of heme's interference in the coagulation system as compiled in this thesis (cf. Chapter 4.1), would greatly enrich the network of HemeKG and would also allow for the detailed analysis of other suggested pathways in heme-driven blood coagulation activation in the future. Overlay of the expanded HemeKG with knowledge from hemolytic disorders could help to differentiate heme-triggered effects from others. A similar approach has been used in the course of the present thesis to compare changes on the molecular level in COVID-19 with heme-triggered effects in hemolysis, suggesting a higher risk for a more severe disease progression of patients with preexisting hemolytic conditions due to similar effects on inflammation, the complement and the blood coagulation system (cf. Chapter 4.4). In addition, an interaction of heme with proteins that support the virus entry into host cells is conceivable based on the data gained in this study. Detailed investigations are required to explore the suggested interactions. As already suggested by others, hemolysis (e.g., AIHA) might occur in COVID-19 infections as well, however, this is currently controversially discussed.[460–463]

In addition to the aforementioned chapters, the present thesis deals with an endogenous natural blood coagulation inhibitor, which was identified and characterized as a heme-regulated protein, i.e. activated protein C (cf. Chapter 4.2). APC binds heme with an affinity in the nanomolar range (K_D ~400 nM) and, thus, with higher affinity than fibrinogen (K_D ~3300 nM) and lesser affinity than FVIIIa (K_D ~1.9 nM) (Table 2). Two highly probable heme-binding sites within the serine protease domain were identified, which are based on a YH- and a HXH-motif. A YH-motif has been already identified in IL-36α as one of the two heme-binding sites and the HXH-motif has recently been suggested to play an important role for heme binding to proteins beyond the common CP-motif.[234,435] *In silico* studies supported the possibility of heme binding to both sites, which resulted in a conformational change of the protein with the stabilization of the light chain. In a pure assay system, heme inhibited the amidolytic activity of APC towards a peptide substrate, which might be due to the heme binding to the YH-motif. This motif is very close to the catalytic triad of APC. These results might correlate with the routinely monitored, lower amidolytic activity of protein C in hemolysis. The conformational change and the binding of heme at the interaction site of APC's natural substrates FVa and FVIIIa results in an impaired anticoagulant function of APC. Heme was even capable of neutralizing the anticoagulant activity of APC in its entirety (concentration-dependent), leading to fast clotting of plasma.

Surprisingly, already without preincubation, an inhibition of APC's anticoagulant activity by heme was observed, supporting fast association of the complex and thus suggesting a rapid intervention in acute hemolytic situations. Furthermore, the formation of the APC-heme complex increases the peroxidase-like activity of heme (~512%; normalized to heme), which might promote prothrombotic reactions *in vivo* as well. While the anticoagulant function of APC is impaired in the presence of heme, its cytoprotective role was not affected. APC even protected endothelial cells from heme-induced loss of permeability, as shown with a molar excess of heme of 12000:1 (heme:APC). The heme-triggered effects on endothelial cells seems to be one of the initial events under hemolytic conditions. To what extent the protective role of APC might play a role under these pathophysiological conditions is not known yet.

In the future, a systematic analysis of coagulation proteins for their heme-binding potential should follow, in order to decipher the complete heme-binding capacity of all blood coagulation proteins. In addition, this characterization might help to derive a potential temporal order of heme binding by considering plasma levels, heme-binding affinity and kinetics. Taken these criteria into account (as also suggested and partially proven for the natural heme-scavenging system; cf. Chapter 2.2), the current state of knowledge might allow for the assumption that although the heme-binding affinities of FVIIIa and APC are much higher than the one of fibrinogen, heme would initially bind to fibrinogen due to the much higher plasma concentration (~100 to >1000-fold). Thus, heme transfer from fibrinogen to APC might be conceivable. Through the direct interaction with these proteins, heme is capable of further potentiation of its procoagulant effects beyond the activation of cellular components. Inflammation processes, the complement system and the blood coagulation system are inevitably interconnected, and all of these processes involve heme-regulated proteins, consequently all of the plasma proteins need to be considered to derive any relations and binding hierarchies. However, the final biological response and reaction remains complex, since acute situations of hemolysis and the associated processes probably change the acute protein plasma levels, which needs to be considered as well.

In conclusion, the present thesis compiles the current knowledge on heme-triggered coagulation activation and adds knowledge on both, the level of heme-induced signaling pathways as well as direct heme-protein interactions. Apart from APC, so far unknown potentially heme-regulated proteins of the blood coagulation system should be analyzed in the future, in order to allow for a more complete understanding of potential interrelations. The range of the effects of heme under hemolytic conditions, considering TLR4 signaling pathway, revealed that TLR4-mediated signal transduction seems to be not only responsible for the proinflammatory role of heme, but partially for its prothrombotic impact. The established network HemeKG requires further enrichment with more experimental data,

which will support a more detailed investigation of heme-triggered signaling in general and concerning comorbidities, as has been demonstrated by the comparison of heme and COVID-19 pathologies.

Further investigations are required to complete our understanding of heme as a procoagulant molecule in hemolytic conditions, which is already evident from the prothrombotic states of patients with hemolytic disorders and needs to be considered in respective treatment strategies.

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Abbreviations

A

ACE2	angiotensin-converting enzyme 2
ADAMTS13	a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
aHUS	atypical hemolytic uremic syndrome
AIHA	autoimmune hemolytic anemia
Akt	protein kinase B
ALAS1	δ-aminolevulinate synthase 1
α1-MG	α1-microglobulin
α1-PI	α1-protease inhibitor
APC	activated protein C
ApoER2	apolipoprotein E receptor 2
AT	antithrombin
ATP	adenosine triphosphate

B

Bach1	BTB and CNC homology 1 (transcription regulator protein)

C

C1q	complement component 1q
C3	complement component 3
C3a	complement component 3a
C3b	complement component 3b
C3dg	complement component 3dg
C5	complement component 5
C5a	complement component 5a
Ca^{2+}	calcium(II) ions
CCL2	chemokine (C-C motif) ligand 2 = MCP-1
CCL3	chemokine (C-C motif) ligand 3 = MIP-1α
CCL5	chemokine (C-C motif) ligand 5 = RANTES
CD11b	cluster of differentiation 11b
CD14	cluster of differentiation 14
CD47	cluster of differentiation 47
CD71	cluster of differentiation 71 = TFR
CD91	cluster of differentiation 91 = LRP1
CD163	cluster of differentiation 163
CLEC2	C-type lectin-like receptor 2
CO	carbon monoxide
COVID-19	coronavirus disease of 2019
CPR	NADPH-cytochrome P450 reductase
CRP	C-reactive protein
*cw*EPR spectroscopy	continuous wave electron paramagnetic resonance spectroscopy
CXCL1	chemokine (C-X-C motif) ligand 1
CXCL2	chemokine (C-X-C motif) ligand 2
Cys	cysteine

D

DAF	decay-accelerating factor (=CD55)
DAMP	damage-associated molecular pattern
DGCR8	DiGeorge critical region 8
DIC	disseminated intravascular coagulation
2,3-DPG	2,3-diphosphoglycerate
DPP8	dipeptidyl peptidase 8
DVT	deep vein thrombosis

E

EPCR	endothelial cell protein C receptor
ER	endoplasmatic reticulum
ERK	extracellular signal-regulated protein kinase

F

FA1	fatty acid binding site 1
FA1B	fatty acid binding site 1B
FDA	food and drug adminstration
FI	fibrinogen
FIa	fibrin
FII	prothrombin
FIIa	thrombin
FIII	tissue factor
FV	proaccelerin, factor V
FVa	accelerin, activated factor V
FVII	proconvertin, factor VII
FVIIa	activated factor VII
FVIII	antihemophilic factor A, factor VIII
FVIII	activated factor VIII
FIX	antihemophilic factor B, Christmas-factor, factor IX
FIXa	activated factor IX
FX	Stuart-Prower factor, factor X
FXa	activated factor X
FXI	plasma thromboplastin antecedent, factor XI
FXIa	activated factor XI
FXII	Hageman-factor, factor XII
FXIIa	activated factor XII
FXIII	fibrin-stabilizing factor, factor XIII
FXIIIa	activated factor XIII

G

gC1q	heterotrimeric globular head domain of human C1q
Glu	glutamic acid
GM-CSF	granulocyte-macrophage colony-stimulating factor
GP	glycoprotein
G6PD	glucose-6-phosphate de-hydrogenase
GPI	glycosylphosphatidylinositol
GSH	glutathione

H

HbF	hemoglobin F
HbS	hemoglobin S
HDL	high-density lipoprotein
His	histidine
HlyC	hemolysin C
HO	heme oxygenase
HO-1	heme oxygenase 1
HO-2	heme oxygenase 2
HMWK	High-molecular weight kininogen
HRG-1	heme responsive gene 1 protein
HSA	human serum albumin
HSP27	heat shock protein 27

I

ICAM-1	intracellular adhesion molecule 1
iC3b	inactivated complement component 3b
ICH	intracerebral hemorrhage
IFNγ	interferon γ
IgA	immunoglobulin A
IgG	immunoglobulin G
IL-1β	interleukin-1β
IL-1Ra	interleukin-1 receptor antagonist
IL-6	interleukin-6
IL-8	interleukin-8
IL-36α	interleukin-36α
IL-36β	interleukin-36β
IL-36γ	interleukin-36γ
IRAKs	IL-1 receptor-associated kinase proteins

J

JNK	c-Jun N-terminal kinase

K

KC	keratinocytes-derived chemokine
K_D	dissociation constant

L

LC3	microtubule-associated protein light chain
LDL	low-density lipoprotein
LPS	lipopolysaccharide
LRP1	LDL receptor-related protein 1 = CD91
LTB4	leukotriene B4
Lu/BCAM	Lutheran/basal cell-adhesion molecule

M

MAC	membrane attack complex =C5b9
MAC-IP	MAC-inhibitor protein = CD59
MAPK	mitogen-activated protein kinase
MCP	membrane cofactor protein

MD2	myeloid differentiation factor 2
MMP8	metalloproteinase 8
MMP9	metalloproteinase 9
MV	microvesicle
MyD88	myeloid differentiation primary response 88

N

NADPH	nicotinamide adenine di-nucleotide phosphate
NETs	neutrophil extracellular traps
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
NMR spectroscopy	nuclear magnetic resonance spectroscopy

O

OATP2	organic anion transporting polypeptide 2

P

PAI-1	plasminogen activator inhibitor-1
PARs	protease-activated receptors
Per2	period circadian protein homolog 2
PIGA	phosphatidylinositol glycan A
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLCγ	phosphoinositide phospholipase Cγ
PNH	paroxysmal nocturnal hemo-globinuria
PS	protein S

R

RAGE	receptor for advanced glycation end products
RBC	red blood cell = erythrocyte
RES	reticuloendothelial system
ROS	reactive oxygen species
rRaman spectroscopy	resonance Raman spectroscopy

S

sCD40L	soluble cluster of differentiation 40 ligand
SFXN	sideroflexin
Syk	spleen tyrosine kinase

T

TAFI	thrombin-activatable fibrinolysis inhibitor
TF	tissue factor
TFR	transferrin receptor 1 = CD71
TIRAP	Toll/IL-1 receptor domain-containing adapter protein
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4

TLRs	Toll-like receptors
TMA	thrombotic microangiopathy
TMPRSS2	transmembrane protease serine 2
TNFα	tumor necrosis factor α
t-PA	tissue-type plasminogen activator
Trp	tryptophan
Tyr	tyrosine

U

UL-VWF	ultra-large von Willebrand factor
UV/vis spectroscopy	ultraviolet/visible spectroscopy

V

Val	valine
VCAM-1	vascular cell adhesion molecule 1
VLDL	very low-density lipoprotein
VOC	vasoocclusive crisis
VWD	von Willebrand disease
VWF	von Willebrand factor

W

WBC	white blood cell/leukocyte

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Publications

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   ** equal contribution*

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1. **Hopp, M.-T.** (**2017**) Impact of heme on proteins of the blood coagulation cascade. *Retreat FOR1738*, Bonn, Germany.

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1. Imhof, D., **Hopp, M.-T.**, Mai, S., Wißbrock, A., Hamedani, N. S., Pötzsch, B. (**2017**) Heme-regulated proteins within the blood coagulation cascade? Insights into the molecular basis of protein binding to free heme. *International Society on Thrombosis and Haemostasis (ISTH) Congress*, Berlin, Germany.

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