The prothrombotic state in paroxysmal nocturnal hemoglobinuria: a multifaceted source

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ABSTRACT

Paroxysmal nocturnal hemoglobinuria is a rare acquired hematologic disorder, the most serious complication of which is thrombosis. The increased incidence of thrombosis in paroxysmal nocturnal hemoglobinuria is still poorly understood, but unlike many other thrombotic disorders, predominantly involves complement-mediated mechanisms. This review article discusses the different factors that contribute to the increased risk of thrombosis in paroxysmal nocturnal hemoglobinuria. Paroxysmal nocturnal hemoglobinuria leads to a complex and multifaceted prothrombotic state due to the pathological effects of platelet activation, intravascular hemolysis and neutrophil/monocyte activation. Platelet and endothelial microparticles as well as oxidative stress may play a role. Impaired fibrinolysis has also been observed and may be caused by several mechanisms involving interactions between complement activation, coagulation and fibrinolysis. While many factors may affect thrombosis in paroxysmal nocturnal hemoglobinuria, the relative contribution of each mechanism that has been implicated is difficult to quantify. Further studies, including novel *in vivo* and *in vitro* thrombosis models, are required in order to define the role of the individual mechanisms contributing to thrombosis, impaired fibrinolysis and clarify other complement-driven prothrombotic mechanisms in paroxysmal nocturnal hemoglobinuria.

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare hematologic disorder of multipotent hematopoietic stem cells. It is caused by an acquired mutation in the X-linked phosphatidylinositol glycan class A gene (*PIG-A*), causing stem cell progeny (mature blood cells) to lack complement regulatory proteins and exposing them to complement attack.[1] Thromboembolic events are the most common cause of morbidity and mortality in PNH and account for 40-67% of deaths; 40% of patients having suffered an event before diagnosis and 29-44% of patients suffering at least one event throughout the course of their disease.[2] Despite such a large role in the burden of the disease, the mechanism behind the development of thrombosis is poorly understood, highlighting the importance of thrombosis management in PNH patients and elucidating more information regarding the nature of the thrombotic event.[3] Multiple proposed mechanisms behind the increased incidence of thrombosis include a prothrombotic state in conjunction with platelet abnormalities and impaired fibrinolysis.[3,4] The review herein will discuss changes to the hemostatic system in PNH, and highlight areas that require future research in the prothrombotic processes involved in PNH.

Paroxysmal nocturnal hemoglobinuria pathophysiology

The incidence of PNH is estimated at 0.1–0.2/100,000 persons per year.[5] PNH is

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caused by an acquired inactivating mutation of the *PIG-A* gene located on the X chromosome. The *PIG-A* gene codes for an enzyme involved in the formation of the N-acetylglucosaminyl phosphatidylinositol biosynthetic protein which is necessary for the first step in the biosynthesis of glycosylphosphatidyinositol (GPI) anchors.[6] GPI, a glycolipid moiety, anchors numerous proteins to the cell surface, with more than 12 GPI-anchored proteins (GPI-APs) located on hemopoietic cells.[1] Studies have shown the presence of a small number of GPI anchor deficient cells in the blood of healthy controls as well as in patients with PNH.[7] This implies that the presence of the *PIG-A* mutation alone is not sufficient to allow the PNH clone to dominate. The process behind the clonal expansion of the *PIG-A* mutated stem cells in PNH patients is not fully understood. Two mutually cooperative hypotheses exist to explain the clonal expansion of PNH cells; one involves immune selection-mediated expansion and the second predicts that dominant PNH clones acquire a growth advantage.[8] The UL16 binding protein 1 (ULBP1), a stress-induced ligand for the NKG2D receptor, is a GPI-linked glycoprotein thought to be lost on PNH stem cells. Loss of ULBP1 may prevent their destruction by NKG2D$^+$ lymphocytes allowing for immune-escape from ULBP-NKG2D engagement in the bone marrow.[9] *PIG-A* mutant cells have been demonstrated to be less sensitive to T lymphocytes and, along with leukemic cells with the same mutation, possess increased resistance to natural killer cells.[9,10] The growth phenotype is thought to be due to the observed upregulation of the early growth response factor 1 gene (*EGR-1*) and ectopic expression of the high mobility transcription factor coding genes *HMGA2* has been reported in a few cases.[11]

The two most significant GPI-APs thought to play a major role in the pathophysiology of PNH are complement regulatory proteins CD55 and CD59.[12] CD55 interacts with C4b and C3b and interferes with their ability to analyze the conversion of C2 and Factor B to active C2a and Bb, thus preventing the formation of C4b2a and C3bBb (both forms of C3 convertase). CD59 interacts directly with the membrane attack complex (MAC), formed at the end of the immune complement cascade, preventing pore formation and cell lysis.[13] The lack of these complement regulatory proteins allows for complement attack leading to erythrocyte lysis, platelet activation and loss of thrombotic modulators on granulocytes, causing many of the symptoms of PNH.[14-16] Exacerbations, or 'paroxysms', are sudden increases in symptoms, most noticeably hemoglobinuria and anemia, caused by infection or other inflammatory stimuli.[17] Eculizumab, a monoclonal antibody inhibiting C5 cleavage, has been shown to significantly reduce the symptoms of PNH as well as associated morbidity and mortality, and is currently the only licensed treatment for PNH patients.[18]

Clinical presentation

Patients may present with 'classical' PNH characterized by clinical and laboratory evidence of intravascular hemolysis with no clinical evidence of an underlying bone marrow disorder. Others have evidence of hemolytic PNH as well as clinical evidence of bone marrow disorder, such as myelodysplasia or aplastic anemia. A further group may be defined as 'subclinical PNH' where a small proportion of PNH cells are found but with no evidence of hemolysis or thrombosis.[19] The presence of PNH cells is identified using flow cytometry, determining the proportion of GPI negative granulocytes, monocytes and erythrocytes.[19] PNH red blood cells can be labeled type I, II or III; type I cells have normal expression of GPI-APs, type II have partial deficiency and type III lack all GPI-APs.[20]

The clinical manifestations are variable; intravascular hemolysis, thrombosis and anemia are significant, however, other symptoms may be present.[1] Schrezenmeier *et al.* analyzed 1610 patients and showed the proportion of symptoms, such as fatigue (80%), dyspnea (64%), hemoglobinuria (62%), abdominal pain (44%), chest pain (33%) and impaired renal function (14%), with only 16% of patients having a history of thrombotic events.[21] However, Hill *et al.* have shown that the presence of subclinical thrombosis is significantly underestimated.[22]

The ongoing effect of intravascular hemolysis, as previously described, is responsible for causing most of the symptoms in PNH (Figure 1). Intravascular hemolysis results in the release of free hemoglobin which is normally cleared by haptoglobin, hemopexin and the scavenger CD163. These clearing mechanisms are overwhelmed in PNH and lead to the accumulation of high levels of free hemoglobin in the plasma, resulting in the scavenging and depletion of nitric oxide (NO).[23] The subsequent excess of hemoglobin leads to the visible hemoglobinuria, while the depletion of NO, a potent vasodilator, results in vasoconstriction, decreased regional blood flow and muscular contraction, causing chest and abdominal pain, amongst other symptoms.[3] Moyo *et al.* reported significant differences in the proportion of PNH cells in patients with symptoms of abdominal pain, hemoglobinuria and esophageal spasm (causing chest pain and dysphagia). The mean proportion of PNH granulocytes in patients with these symptoms was at least twice that of patients who did not possess these clinical manifestations.[24]

As previously described, thrombosis is the most serious complication associated with PNH. Thrombotic events are reported to be of venous origin in 85% of cases, arterial in 15% of cases and involve more than one site at the same time in 20.5% of cases.[5] Thrombosis can occur at any site, with deep vein thrombosis, pulmonary embolism, myocardial infarction or cerebral vascular attack all commonly observed complications.[25] There appears to be an increased incidence of thrombosis at atypical sites, such as the hepatic vein resulting in Budd-Chiari syndrome, occurring in 40-44% of PNH patients, in addition to thrombosis in the vasculature of the central nervous system, mesenteric, dermal veins and the cavernous sinus.[26] The proportion of PNH cells and clone size has also been associated with thrombotic complications. Hall *et al.* demonstrated that in patients with a proportion of PNH granulocytes greater than 50%, the 10-year risk of thrombosis was 44%, but in patients with a proportion of PNH granulocytes less than 50% the risk was 5.8%.[27] Through logistical regression, Moyo *et al.* calculated the increase in odds ratio for thrombosis to be 1.64 for each 10% increase in the proportion of PNH cells.[24] These findings correlate with other studies which have shown that the occurrence of thrombosis is noticeably elevated in PNH patients with a proportion of PNH cells as low as 10% when compared to normal population controls.[28,29]

Platelet activation

The mechanisms behind thrombus formation in PNH are complex and subject to continued research. Interactions between the complement system, platelets and coagulation likely explain some of the increased risk of thrombosis. Due to the multifactorial and variable nature of the disease, it is

likely that a combination of several factors may contribute to the increased incidence of thrombus formation and associated mortality (Figure 2).

Platelets have been reported to play a significant role in the formation of thrombus in PNH patients, by both contributing to a prothrombotic state and initiating clot formation.[30] One would expect that due to the deficiency of CD55 and CD59, lysis of platelets occurs and contributes to thrombocytopenia. However, this is not the case, as the lifespan of platelets in PNH patients is normal.[31] Rather than complement resulting in the lysis of platelets, an intrinsic mechanism of adaption and resistance to complement attack has been observed which subsequently contributes to the prothrombotic state.[32] It has been shown that upon increased deposition of MAC (C5b-9) on the membrane of platelets, rather than lysis, complement accumulation on the platelet surface triggers morphological changes.[15] The loss of platelet membrane phospholipid asymmetry through the action of an adenosine triphosphate (ATP)-dependent enzyme, gelsolin, aminophospholipid translocase, lipid scramblase and calpain allow for cytoskeletal and phospholipid bilayer changes.[33] The now activated platelet secretes α-granules, and in conjunction with membrane depolarization, α-granules fuse with the platelet membrane.[34] This results in exocytosis of the vesiculated MAC and the production of prothrombotic platelet-derived microparticles (PMPs).[15]

Platelet-derived microparticles

The exact role of PMPs is not fully understood, however, they are considered to play a role in the generation of a prothrombotic state in PNH.[15] Three key hypotheses for the prothrombotic nature of PMPs have been identified.[35] First, platelet microparticles are formed by platelets upon activation, therefore their membranes possess the same prothrombotic properties as the activated platelet membrane.[36] Second, PMPs can bind clotting cascade components, such as activated factors V (Va) and VIII (VIIIa); furthermore, the densities of these protein binding sites on PMPs appear to exceed those on activated platelet membranes.[37,38] Finally, when isolated PMPs are added back to platelet free pooled plasma without the addition of coagulation activators, they trigger thrombin generation, demonstrating that microparticles generated *in vivo* can stimulate coagulation.[39] Sinauridze *et al.* estimated that PMP membranes have a 50- to 100-fold higher specific procoagulant activity than activated platelets.[35]

PMPs have been shown to express many of the following membrane binding protein complexes which are normally observed on activated platelets: glycoprotein Ib (GPIb), which binds von Willebrand factor (VWF) initiating formation of the platelet plug;[40] platelet endothelium adhesion molecule (PECAM-1), an immunoglobulin superfamily member involved in leukocyte transmigration, angiogenesis, and integrin activation;[41] the integrin glycoprotein IIb/IIIa (GpIIb-IIIa) or $\alpha_{IIb}\beta_3$, a receptor for fibrinogen and VWF, further aiding platelet aggregation and plug formation;[42] and P-selectin, a cellular adhesion molecule which exacerbates symptoms *via* a feedback loop through continued stimulation of the alternative pathway, initiating activation of the classical pathway as well as stimulating further platelet aggregation.[43] The expression of membrane proteins involved in thrombus formation on platelet microparticles suggests an ability to contribute to the prothrombotic state.

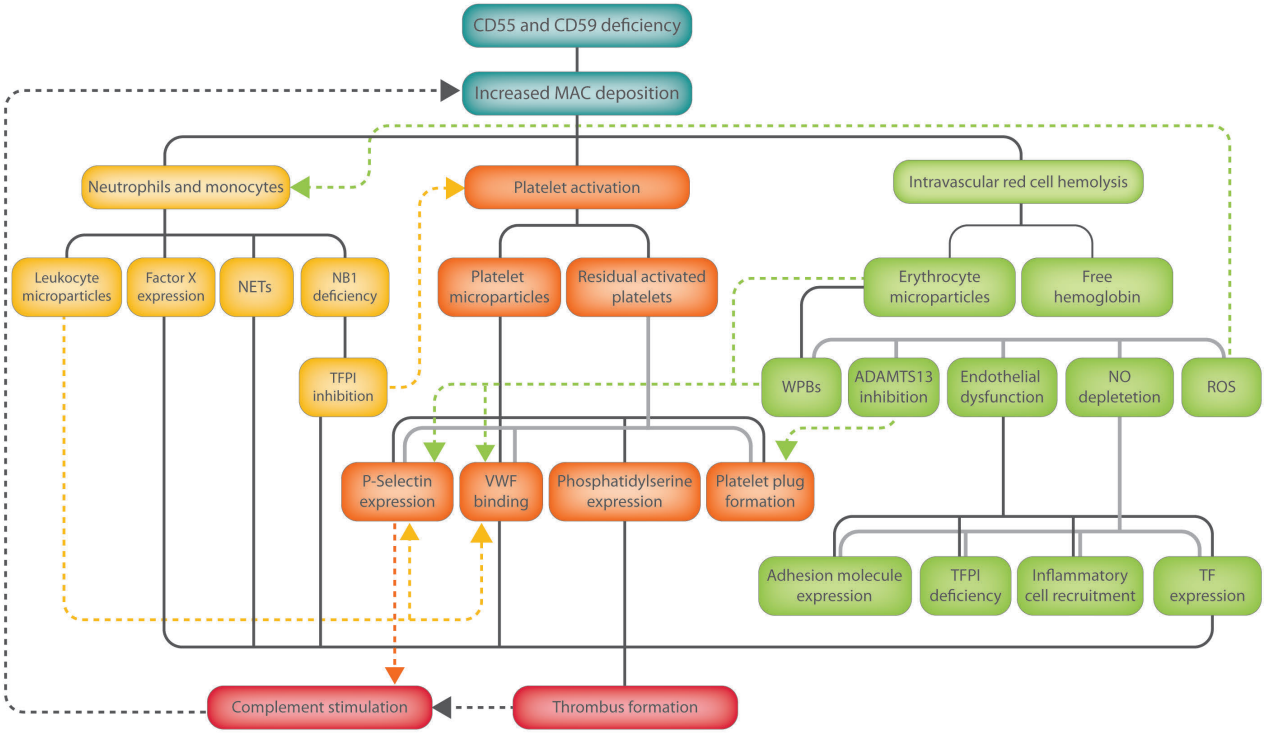

Figure 1. Summary of the multiple factors thought to contribute to the prothrombotic state in paroxymal nocturnal hemoglobinuria (PNH) and interaction. Further details are provided in the text. MAC: membrane attack complex; NET: neutrophil extracellular traps; TFPI: tissue factor pathway inhibitor; VWF: von Willebrand factor; WPB: Weibel-Palade bodies; NO: nitric oxide; ROS: reactive oxygen species; TF: tissue factor.

However, further studies are necessary to analyze and quantify their specific role in PNH-induced risk of thrombosis.

Phosphatidylserine is expressed on the surface of platelet-derived microparticles as a result of MAC binding-induced morphological changes.[44] Phosphatidylserine is normally confined to the inner leaflet of the platelet membrane, however, it is translocated to the outer leaflet due to the action of the lipid scramblase enzyme and exposed as a result of platelet activation.[33] Phosphatidylserine interacts *via* positively charged calcium ions with negatively charged γ-carboxyglutamic acid (GLA) domains in vitamin K-dependent clotting factors, e.g. VII (FVII), IX, X, and prothrombin.[45] This catalyzes the formation of the procoagulant enzyme complexes prothrombinase (VaXa) and tenase (VIIIaIXa),[37] and allows for the accelerated conversion of prothrombin to thrombin and stimulation of the coagulation cascade.[44] Coagulopathy has been observed in patients with Castaman defect and Scott syndrome, and is thought to result from defects in the action of scramblases to translocate phosphatidylserine to the membrane surface.[46,47]

Residual activated platelets

The prothrombotic properties of residual activated platelets (platelets post microparticle production) is still a matter of discussion. Activated platelets in PNH patients have been shown to possess greater than ten times the factor V binding sites compared with those from normal controls.[16] Activated platelets have been shown to promote thrombus formation through neutrophil interaction, resulting in the release of serine proteases and nucleosomes, activating Factor X.[48] Like their exocytosed microparticles, activated platelets also express P-selectin, which is thought to further stimulate the complement pathway.[49] Surprisingly, a study by Grünewald *et al.* found evidence of hyporeactive platelets in PNH.[50] It is possible that the failure of activated platelets to bind fibrinogen, VWF and to aggregate is due to receptor GpIIb-IIIa complexes in proximity to MAC pores becoming uncoupled from the intracellular transduction mechanisms normally involved in their activation.[37] This observation was consistent with the findings of Gralnick *et al.*, who reported variable amounts of platelet activation and further observed reduced VWF binding.[30] Grünewald *et al.* hypothesized a mechanism of dual causality responsible for platelet hyporeactivity.[50] One mechanism is hyperstimulation of platelets due to sustained complement attack.[16,37,50] Chronic hyperstimulation of the coagulation system was hypothesized to further downregulate the activity of activated platelets.[4,50] This highlights the complex variable nature of thrombosis in PNH, suggesting that platelets post activation possibly have a diminished role in PNH-induced thrombosis in comparison to other thrombotic diseases.

Hemolysis

As well as contributing to a wide array of symptoms in PNH, hemolysis is thought to contribute to a prothrombotic state, but its role is becoming increasingly scrutinized.[51] Erythrocytes have been reported to produce microparticles as a result of MAC-induced apoptosis.[52] Some microparticles observed in PNH have indeed been confirmed to originate from erythrocytes; however, Hugel *et al.* reported that this was not 'to a significant extent' while 'very high levels' of microparticles of platelet origin were detected.[15] Two studies have concluded that the level of erythrocyte microparticles produced in PNH patients was similar to healthy controls;[15,53] hence, it is possible that the contribution of erythrocyte microparticles to the prothrombotic state in PNH is only minimal.

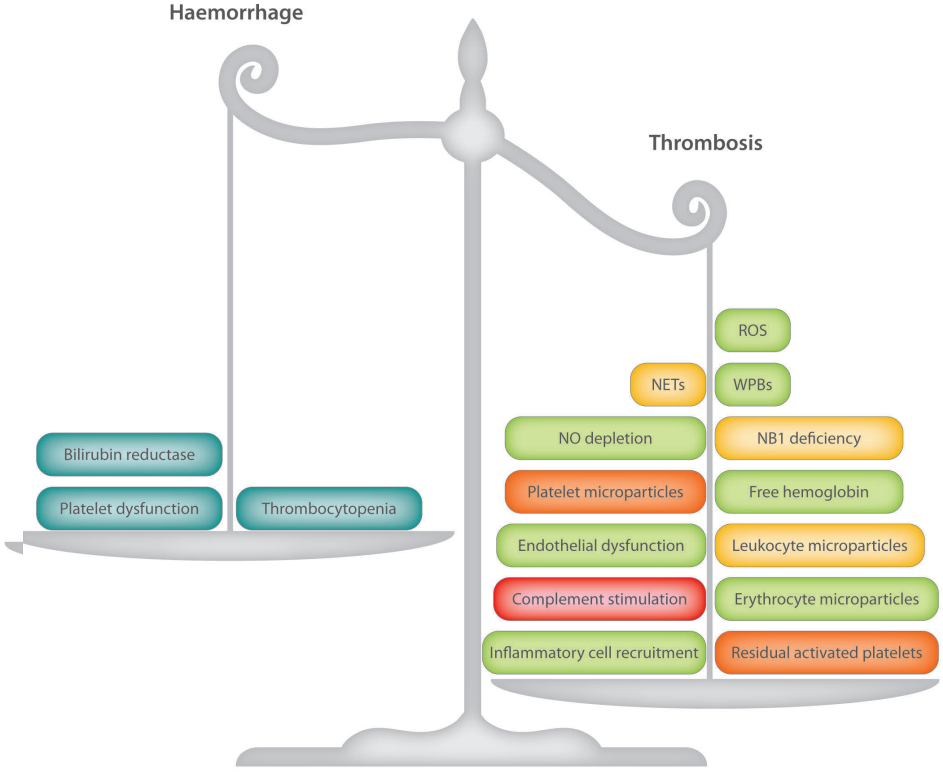

Figure 2. Hemostasis mechanisms in paroxymal nocturnal hemoglobinuria (PNH) patients are imbalanced towards thrombosis. NET: neutrophil extracellular traps; WPB: Weibel-Palade bodies; ROS: reactive oxygen species; NO: nitric oxide.

Free Hemoglobin and Endothelial Dysfunction

Excess free hemoglobin is a further possible mechanism that may underpin the prothrombotic state in PNH. Upon intravascular hemolysis, free hemoglobin is rapidly bound to the serum protein haptoglobin expressed on monocytes/macrophages forming a complex which is then endocytosed and degraded by CD163.[24,54,55] The oxygen binding component of hemoglobin, ferrous heme, can be oxidised to ferric heme, resulting in rapid binding to hemopexin.[56] The resulting reaction has vasodilatory, antiproliferative, anti-inflammatory, and antioxidant properties through the release of carbon monoxide, the biliverdin metabolite biliverdin reductase, and the uptake of anti-inflammatory interleukin-10 and heme oxygenase into circulating monocytes.[54,57] The scavenging mechanisms described above can become saturated resulting in increased levels of free hemoglobin in the circulation, leading to a prothrombotic state in addition to other symptoms.[23,24]

There is increasing evidence supporting possible prothrombotic effects of free hemoglobin on platelets and the vascular endothelium.[58] A recent study by Belcher *et al.* showed that heme rapidly stimulates the release of Weibel-Palade bodies (WPBs) from the vascular endothelium.[59] Degranulation of WPBs releases VWF and P-selectin onto the surface of endothelial cells, stimulating coagulation and the complement cascade.[59] *In vivo* studies have demonstrated that the infusion of crosslinked hemoglobin increased platelet aggregation and adhesion to the endothelium of an injured vessel wall.[60] Free hemoglobin has been observed to directly bind to VWF exposed on the endothelium which increases its affinity for the glycoprotein Ib (GPIb) receptor on the surface of platelets.[61] Conjointly, the addition of free hemoglobin to human serum causes inhibition of the VWF cleaving protease ADAMTS13, an enzyme critical in limiting platelet thrombus formation.[62,63] Heme administration in healthy volunteers has been demonstrated to cause thrombophlebitis, vascular inflammation and obstruction.[64]

Patients with PNH have also been observed to possess increased levels of endothelial-derived microparticles (EMPs).[40,65] GPI-deficient monocytes are thought to release microparticles rich in tissue factor (TF) upon complement damage.[66] Uptake of monocyte-derived microparticles concomitantly increased endothelial TF expression while producing EMPs. Two separate studies have observed increased levels of EMPs in patients with PNH.[40,67] The number of EMPs produced relative to PMPs is thought to be small, and as such its contribution to the prothrombotic state is minimal.[53] Further procoagulant effects of endothelial cells result from prolonged exposure to free heme.[68] Endothelial exposure to heme induces tissue factor expression, which can initiate coagulation, and also activates expression of intracellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule (VCAM1), and E-selectin.[69] The now activated endothelial cells recruit inflammatory cells, promoting thrombus formation at the vessel wall.[23] This can be further enhanced by pro-inflammatory cytokines and chemokines which have been observed as being over-expressed in other hemolytic disorders.[70] It is still unknown whether bone marrow-derived endothelial cells in PNH patients harbor the GPI-AP deficiency.[66] Tissue factor pathway inhibitor (TFPI) inhibits tissue factor and therefore coagulation.[71] It is predominantly produced by the endothelium (85%), however, platelets, monocytes and plasma are other sources.[66,71] TFPI is expressed in two isoforms, TFPI-α and TFPI-β.[72] It is still disputed which TFPI isoform is the most abundantly expressed isoform, however TFPI-β, a GPI-AP expressed on ECs, is thought to exert 80% of anticoagulant activity.[72,73] Possible deficiency of the GPI anchor of TFPI-β in PNH may therefore reduce the anticoagulant properties of the endothelium in PNH and contribute to thrombus formation.[74,75]

Anti-thrombin is enhanced by binding to the heparan sulphate receptor, a GPI-linked protein expressed on endothelial cells and hypothesized to be lost in patients with PNH.[76] As the heparin sulphate receptor is GPI-linked, its loss is thought to contribute to a prothrombotic state. No studies have fully investigated the significance of the heparan sulphate receptor, however, a compensatory mechanism has been suggested after there was no change in fibrin deposition in animal studies of heparan sulphate deficiency.[77]

Reactive oxygen species

Free hemoglobin has also been demonstrated to produce reactive oxygen species (ROS) *via* two mechanisms:[23] the amphipathic heme interacts with the phospholipid membrane, and *via* the Fenton reaction catalyzes the production of ROS,[78] and extracellular hemoglobin autoxidizes to methemoglobin catalyzed by peroxidase enzymes which further generates ROS.[79] A study by Amer *et al.* has also shown that PNH cells have a higher oxidative status when compared to normal cells.[80] It is not clear if this is as a result of free hemoglobin, platelet hyperactivity (as previously discussed) or a pre-existing defect in PNH cells that exacerbates these pathologies, thus, further research into this area is necessary. The formation of ROS is well-documented to produce phospholipid disorganisation, induce cytotoxicity and promote inflammation,[23,81] and studies have also shown that ROS can directly enhance platelet activation.[82] The Fenton reaction also activates protein kinase C as well as ROS which have been demonstrated to activate platelets.[82]

Neutrophils and monocytes

Neutrophils have also been reported to contribute to the prothrombotic mechanisms in PNH.[23,56] ROS produce neutrophil extracellular traps (NETs), exposed extracellular chromatin with peripherally attached enzymes.[59] Extracellular chromatin has been observed as a structural component in deep vein thrombi as well as contributing to the pathogenesis involved in their formation.[83] Studies have found that histones, which are also released from NETs, increase thrombin generation and platelet activation by impairing the activation of protein C.[84] The impairment of protein C activation in turn prevents the inactivation of clotting FV and FVIII which, as examined previously, form the prothrombinase and tenase complexes, respectively, on platelets.[85] Recent studies have demonstrated a specific link between NETs and the formation of venous thrombosis. This mechanism may explain the high prevalence of thrombi in veins at atypical sites and warrants further study in PNH.[83,84] The membrane attacks complex formation on the surface of monocytes and neutrophils and is also thought to contribute to the procoagulant state in PNH.[3,23] Complement-induced cell activation results in the expression of tissue factor as well as plasminogen activator inhibitor 1, contributing to thrombus

formation while impairing fibrinolysis (discussed below).[86] Proteinase 3 (PR3), an enzyme thought to reduce thrombin-induced platelet activation binds to the GPI-anchored co-factor NB1 (CD177) expressed on neutrophils.[87] A deficiency of NB1 may therefore contribute to platelet activation and exacerbate the procoagulant state through proteolysis of the protein C receptor, degradation of TFPI and upregulation of TF expression on endothelial cells.[88-90]

Prothrombotic feedback mechanisms

Thrombin, the generation of which is increased by many of the mechanisms described above, has been observed to independently activate complement proteins C3 and C5.[91] Plasmin, an enzyme involved in fibrin clot degradation and stimulated by fibrin itself, has also recently been shown to cleave C5.[92] This suggests a feedback mechanism in which thrombin generation, fibrin deposition and fibrinolysis may in turn activate the complement system, which reciprocally leads to more platelets and coagulation activation, exacerbating the thrombotic response (see Figures 1 and 2 for a summary of prothrombotic mechanisms involved in PNH).[93] Plasmin has been shown *in vitro* to initiate the synthesis of platelet activating factor (PAF) from endothelial cells,[94] while a further *in vivo* study has shown a correlation between the concentration of the terminal complement complex (C5b-9) and PAF. This may begin to highlight a mechanism in which the MAC contributes to plasmin-induced synthesis of PAF in endothelial cells, however, it is unclear whether this would contribute to thrombosis or platelet hyporeactivity.[95]

Nitric oxide depletion

Hemoglobin and nitric oxide (NO) bind in an irreversible reaction, the rate of which is suggested to increase by up to 500-600 times as a result of intravascular hemolysis and the loss of heme compartmentalization.[96] Intravascular hemolysis also releases arginase which breaks down L-arginine, the substrate for NO synthesis.[97] NO depletion has been well-established as a potent regulator of smooth muscle tone, causing vascular constriction and contributing to a prothrombotic state.[1,3,56] One study has found a 12-fold increase in the consumption of NO in PNH patients when compared to healthy volunteers in addition to an increase in pulmonary hypertension.[98] As well as having a vasodilatory effect, NO also binds to platelets, causing signal transduction that downregulates the expression of the fibrinogen binding integrin glycoprotein IIb/IIIa, reduces levels of intracellular calcium and inhibits platelet activation.[99,100] A reduction in circulating NO results in further dysregulation of platelets, and, combined with local vasoconstriction can contribute to intravascular thrombosis.[56]

Fibrin clot structure

There is a growing body of evidence in the literature that individuals with an increased risk of thrombosis form fibrin clots with an altered 3-dimensional structure.[101] The altered clot structure is comprised of thinner but more tightly packed fibrin fibers, which, possibly combined with fewer binding sites for plasmin and tissue plasminogen activator (tPA), leads to impaired fibrinolysis. The dense clot structures are more resistant to fibrinolysis due to the increased number of fibers that need to be lysed and a reduced permeation of the lytic enzymes into the denser clot structure.[102] Moreover, clots with densely packed fibers are stiffer and more resistant to mechanical deformation.[103] Due to these structural and functional changes, dense clots with smaller pores are associated with an increased risk of thrombosis. There have been no studies to date that have investigated clot structure in patients with PNH. Abnormal clot structure could be an additional mechanism by which the risk of thrombosis is increased in PNH, deserving further study.

Additionally, the link between PNH and clot structure may be of interest, since complement activation and factors have been associated with effects on fibrin clot structure and function. For example, alternative complement pathway activation has been associated with the production of denser, more tightly packed clots;[104] furthermore, C3 has been shown to be incorporated into the fibrin clot, leading to thinner fibrin fibers and a stiffer clot with increased resistance to fibrinolysis.[105] In addition, MASP-1 has been shown to influence clot formation and activate coagulation Factor XIII, leading to an increased resistance of the clot to fibrinolysis.[106] There may yet be other, unidentified mechanisms by which complement activation may regulate clot structure and function.

High plasma levels of fibrinogen, the molecular precursor to fibrin, are known to affect clot structure.[107] Seregina *et al.* measured fibrinogen levels in PNH patients pre- and post-treatment with eculizumab (n=3), and found no difference from normal controls.[18] This suggests that clot structure is not being modulated by high levels of fibrinogen in PNH patients, but the small sample size means further study is necessary.

Studies have shown that high concentrations of thrombin during fibrin clot formation results in prothrombotic fibrin structure and more stable clots.[108] Surprisingly, one study found lower levels of thrombin generation in PNH patients. However, this may have been partly caused by the fact that thrombin generation in this study was measured in the absence of platelets and endothelial cells, which have been shown to be activated in PNH and enhance thrombin generation, as previously discussed.[75] An alternative study found significantly elevated levels of thrombin generation on endothelial cells in patients with PNH.[67] As examined previously, both activated platelets, PMPs and NETs increase thrombin generation and, therefore, may modulate clot structure through increased thrombin generation. Oxidized red blood cells integrated into fibrin clots have also been observed, enhancing clot stability.[109] The oxidation of fibrin fibers has also been proposed to alter clot structure; however, this has been shown to both impair fibrin formation as well as produce thinner fibers and weaker clots.[110]

The role of microparticles in modulating clot structure is still a matter of dispute. Aleman *et al.* found that while platelet microparticles appeared to have no effect on clot structure, monocyte-derived microparticles supported faster fibrin deposition and a denser, more stable fibrin clot.[111]

Impaired fibrinolysis

Impaired fibrinolysis has been indicated in patients with PNH.[3,4,66] Urokinase-type plasminogen activator receptor (uPAR) is a GPI-AP, and as such is absent from PNH monocytes and granulocytes.[112] This results in increased plasma levels of free uPAR protein, which is thought to compete with the membrane bound uPA receptor, competitively

inhibiting cell-based plasmin generation and therefore contributing to a prothrombotic state in PNH.[113,114] As discussed above, neutrophils, when activated, can express plasminogen activator inhibitor-1, impairing plasminogen and urokinase and therefore inhibiting fibrinolysis. Studies have also observed activated platelets releasing inhibitory proteases, plasminogen activator inhibitor-1 and α2-antiplasmin.[115] More studies are needed to identify further causes of impaired fibrinolysis, whether it be resulting from GPI-AP loss or the hyperactivity of prothrombotic cells.

Animal models

Mouse models have been used in the study of PNH, however it has proven difficult to replicate the thrombotic sequelae resulting from CD55 and CD59 deficiency. Complications arise due to the fact that there are two different CD55 and CD59 coding genes, a and b, respectively, and as a result, isolating their respective phenotypes following knockout has proven challenging.[116,117] Mice also possess a unique transmembrane protein, complement-receptor 1-related gene protein (Crry), a functional homolog of human membrane co-factor protein which plays a critical role in protecting developing fetuses in mice from lethal complement attack. It has only been possible to produce Crry/C3 double knockout mice, which showed evidence of extravascular hemolysis, however, mice erythrocytes and platelets lacking Crry have been demonstrated to be more susceptible to hemolysis.[118,119] Generating PIG-A mutations has proven difficult, as PIG-A deletion in embryonic stem cells is lethal.[120] A PIG-A floxed mosaic mice model was generated with the coexistence of normal and mutated cells mimicking PNH patients, however, the PNH clone failed to clonally expand and produce PNH symptoms.[121] Kellet *et al.* succeeded in creating a model with 100% of red blood cells being GPI-AP negative, however, no symptoms of PNH were observed. Limited knowledge of the clonal expansion mechanism has made it difficult to create animal models in which the thrombotic mechanisms in PNH can be analyzed. Future studies into clonal expansion mechanisms may lead to the development of more appropriate *in vivo* models, enabling the study of the mechanisms of thrombosis in PNH.

Summary and future perspectives

Thrombosis risk is greatly increased in patients with PNH and is the greatest cause of morbidity and mortality in the disease. However, the mechanisms underpinning this are far from clear and are likely to be different from those of other thrombotic disorders. The prothrombotic state in PNH is extremely complex, with many different factors resulting from platelet activation, intravascular lysis and neutrophil/monocyte activation all thought to play a role. Further research is necessary in order to quantify how much each of these factors contribute to the prothrombotic state as well as to analyze their role *in vivo*. The newly hypothesized role of NETs especially warrants investigation, as this may explain the high and sustained incidence of atypical thrombosis in PNH. Impaired fibrinolysis and alterations to clot structure also appear to be hallmarks of thrombotic events, and it is important to determine the role of eculizumab in modifying these. Further identification of GPI-APs involved in clot structure and impaired fibrinolysis, as well as clarification as to whether endothelial cells lack GPI-APs, is necessary in order to understand the complex mechanism of thrombosis in PNH. With PNH research now in the post-eculizumab era, our understanding of the complement-mediated disease processes has improved dramatically, but those underpinning thrombotic complications are still insufficiently understood, despite the many hypothetical mechanisms which have been proposed. Future studies, including those involving animal models of PNH, may help to address this hiatus while simultaneously highlighting similar prothrombotic mechanisms in other, related hemolytic complement diseases. Identifying the factors that most significantly contribute to thrombus formation in PNH would allow for the application of more targeted therapies, potentially minimizing the disease burden and further improving patient outcomes.

References

1. Brodsky RA. Paroxysmal nocturnal hemoglobinuria. Blood. 2015;124(18):2804-2812.
2. Socié G, Mary J, de Gramont A, Rio B, et al. Paroxysmal nocturnal haemoglobinuria: long-term follow-up and prognostic factors. Lancet. 1996;348(9027):573-577.
3. Hill A, Kelly RJ, Hillmen P. Thrombosis in paroxysmal nocturnal hemoglobinuria. Blood. 2013;121(25):4985-4996.
4. Grünewald M, Siegemund A, Grünewald A, et al. Plasmatic coagulation and fibrinolytic system alterations in PNH: relation to clone size. Blood Coagul Fibrinolysis. 2003;14(7): 685-695.
5. Devalet B, Mullier F, Chatelain B, Dogné JM, Chatelain C. Pathophysiology, diagnosis, and treatment of paroxysmal nocturnal hemoglobinuria: a review. Eur J Haematol. 2015;95(3):190-198.
6. Miyata T, Takeda J, Iida Y, et al. The cloning of PIG-A , a component in the early step of GPI-anchor biosynthesis. N Engl J Med. 1994;259(5099):1318-1320.
7. Bessler M, Mason P, Hillmen P, Luzzatto L. Somatic mutations and cellular selection in paroxysmal nocturnal haemoglobinuria. Lancet. 1994;343:951-953.
8. Nakakuma H, Kawaguchi T. Pathogenesis of selective expansion of PNH clones. Int J Hematol. 2003;77(2):121-124.
9. Kawaguchi T, Nakakuma H. New insights into molecular pathogenesis of bone marrow failure in paroxysmal nocturnal hemoglobinuria. Int J Hematol. 2007;86(1):27-32.
10. Hanaoka N, Kawaguchi T, Horikawa K, Nagakura S, Mitsuya H, Nakakuma H. Immunoselection by natural killer cells of PIGA mutant cells missing stress-inducible ULBP. Blood. 2006;107(3):1184-1191.
11. Inoue N, Izui-Sarumaru T, Murakami Y, et al. Molecular basis of clonal expansion of hematopoiesis in 2 patients with paroxysmal nocturnal hemoglobinuria (PNH). Blood. 2006;108(13):4232-6.
12. Schubert J, Roth A. Update on paroxysmal nocturnal haemoglobinuria: on the long way to understand the principles of the disease. Eur J Haematol. 2015;94(6):464-473.
13. Rollins SA, Sims PJ. The complement-inhibitory activity of CD59 resides in its capacity to block incorporation of C9 into membrane C5b-9. J Immunol. 1990;144(9): 3478-3483.
14. Jankowska AM, Szpurka H, Calabro M, et al. Loss of expression of neutrophil proteinase-3: A factor contributing to thrombotic risk in paroxysmal nocturnal hemoglobinuria. Haematologica. 2011;96(7):954-962.
15. Hugel B, Socie G, Vu T, Toti F, et al. Elevated levels of circulating procoagulant microparticles in patients with paroxysmal nocturnal hemoglobinuria and aplastic anemia. Blood. 1999;93:3451-3456.
16. Wiedmer T, Hall SE, Ortel TL, Kane WH, Rosse WF, Sims PJ. Complement-induced vesiculation and exposure of membrane prothrombinase sites in platelets of paroxysmal nocturnal hemoglobinuria. Blood. 1993;82 (4):1192-1196.
17. Hillmen P, Lewis SM, Bessler M, Luzatto L, Dacie J V. Natural history of paroxysmal nocturnal hemoglobinuria. Blood. 1993;333 (19):1253-1258.
18. Seregina EA, Tsvetaeva NV, Nikulina OF, et al. Eculizumab effect on the hemostatic state

in patients with paroxysmal nocturnal hemoglobinuria. Blood Cells Mol Dis. 2015;54(2):144-150.
19. Parker C, Omine M, Richards S, et al. Diagnosis and management of paroxysmal nocturnal hemoglobinuria. Blood. 2005;106 (12):3699-3709.
20. Hill A, Richards SJ, Hillmen P. Recent developments in the understanding and management of paroxysmal nocturnal haemoglobinuria. Br J Haematol. 2007;137(3):181-192.
21. Schrezenmeier H, Muus P, Socié G, et al. Baseline characteristics and disease burden in patients in the international paroxysmal nocturnal hemoglobinuria registry. Haematologica. 2014;99(5):922-929.
22. Hill A, Reid SA, Rother RP, et al. High definition contrast-enhanced MR imaging in paroxysmal nocturnal hemoglobinuria (PNH) suggests a high frequency of subclinical thrombosis. Blood. 2015;108(11):292.
23. L'Acqua C, Hod E. New perspectives on the thrombotic complications of haemolysis. Br J Haematol. 2015;168(2):175-185.
24. Moyo VM, Mukhina GL, Garrett ES, Brodsky RA. Natural history of paroxysmal nocturnal haemoglobinuria using modern diagnostic assays. Br J Haematol. 2004;126 (1):133-138.
25. Hill A, Kelly RJ, Hillmen P. Thrombosis in paroxysmal nocturnal hemoglobinuria. Blood. 2013;121(25):4985-4996.
26. Ziakas P, Poulou L, Rokas G, Bartzoudiss D, Voulgarelis M. Thrombosis in paroxysmal nocturnal hemoglobinuria: sites, risks, outcomes. An overview. J Thromb Haemost. 2007;5(3):642-645.
27. Hall C, Richards S, Hillmen P. Primary prophylaxis with warfarin prevents thrombosis in paroxysmal nocturnal hemoglobinuria (PNH). Blood. 2003;102(10):3587-3591.
28. Hoekstra J, Leebeek FWG, Plessier A, et al. Paroxysmal nocturnal hemoglobinuria in Budd-Chiari Syndrome: Findings from a cohort study. J Hepatol. 2009;51(4):696-706.
29. Fowkes FJI, Price JF, Fowkes FGR. Incidence of diagnosed deep vein thrombosis in the general population: systematic review. Eur J Vasc Endovasc Surg. 2003;25(1):1-5.
30. Gralnick HR McKeown LP, Merryman P, Wilson O, Chu I, Kimball J VM. Activated platelets in paroxysmal nocturnal hemoglobinuria. Br J Haematol. 1995;91:697-702.
31. Devine DV, Rosse WF. Regulation of the activity of platelet-bound C3 convertase of the alternative pathway of complement by platelet factor H. Proc Natl Acad Sci USA. 1987;84(16):5873-5877.
32. Wiedmer T, Esmon CT, Sims PJ. Complement proteins C5b-9 stimulate procoagulant activity through platelet prothombinase. Blood. 1986;123(23):3533-3535.
33. Bevers EM, Comfurius P, Dekkers DWC, Zwaal RFA. Lipid translocation across the plasma membrane of mammalian cells. Biochim Biophys Acta. 1999;1439(3):317-330.
34. Blair P, Flaumenhaft R. Platelet alpha–granules: basic biology and clinical correlates. Blood Rev. 2009;23(4):177-189.
35. Sinauridze EI, Kireev DA, Popenko NY, et al. Plateletmicroparticle membranes have 50- to 100-fold higher specificprocoagulant activity than activated platelets. Thromb Haemost. 2007;97(6):425-434.
36. Sims PJ, Wiedmer T, Esmon CT, Weiss HJ, Shattil SJ. Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. J Biol Chem. 1989;264(29)17049-17057.
37. Sims PJ, Faioni EM, Wiedmer T, Shattil SJ. Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. J Biol Chem. 1989;264(29):17049-17057.
38. Gilbert GE, Sims PJ, Wiedmer T, Furie B, Furie BC, Shattil SJ. Platelet-derived microparticles express high affinity receptors for factor VIII. J Biol Chem. 1991;266 (26):17261-17268.
39. Nieuwland R, Berckmans RJ, Rotteveel-Eijkman RC, et al. Cell-derived microparticles generated in patients during cardiopulmonary bypass are highly procoagulant. Circulation. 1997;96(10):3534-3541.
40. Simak J, Holada K, Risitano AM, Zivny JH, Young NS, Vostal JG. Elevated circulating endothelial membrane microparticles in paroxysmal nocturnal haemoglobinuria. Br J Haematol. 2004;125(6):804-813.
41. Losy J, Niezgoda A, Wender M. Increased serum levels of soluble PECAM-1 in multiple sclerosis patients with brain gadolinium-enhancing lesions. J Neuroimmunol. 1999;99(2):169-172.
42. Schwarz M, Katagiri Y, Kotani M, et al. Reversibility versus persistence of GPIIb/IIIa blocker-induced conformational change of GPIIb/IIIa (alphaIIbbeta3, CD41/CD61). J Pharmacol Exp Ther. 2004;308(3):1002-1011.
43. Del Conde I, Crúz MA, Zhang H, López JA, Afshar-Kharghan V. Platelet activation leads to activation and propagation of the complement system. J Exp Med. 2005;201(6):871-879.
44. Lacroix R, Dignat-George F. Microparticles as a circulating source of procoagulant and fibrinolytic activities in the circulation. Thromb Res. 2012;129(Suppl 2):S27-29.
45. Owens AP, Mackman N. Microparticles in hemostasis and thrombosis. Circ Res. 2011;108(10):1284-1297.
46. Castaman G, Yu-Feng L, Battistin E, Rodeghiero F. Characterization of a novel bleeding disorder with isolated prolonged bleeding time and deficiency of platelet microvesicle generation. Br J Haematol. 1997;96(3):458-463.
47. Weiss HJ, Lages B. Family studies in Scott syndrome. Blood. 1997;90(1):475-476.
48. Massberg S, Grahl L, von Bruehl ML, et al. Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases. Nat Med. 2010;16(8):887-896.
49. Peerschke EI, Yin W, Grigg SE, Ghebrehiwet B. Blood platelets activate the classical pathway of human complement. J Thromb Haemost. 2006;4(9):2035-2042.
50. Grünewald M, Grünewald A, Schmid A, et al. The platelet function defect of paroxysmal nocturnal haemoglobinuria. Platelets. 2004;15(3):145-154.
51. Devalet B, Mullier F, Chatelain B, Dogné J-M, Chatelain C. The central role of extracellular vesicles in the mechanisms of thrombosis in paroxysmal nocturnal haemoglobinuria: a review. J Extracell Vesicles. 2014;3:1-8.
52. Kozuma Y, Sawahata Y, Takei Y, Chiba S, Ninomiya H. Procoagulant properties of microparticles released from red blood cells in paroxysmal nocturnal haemoglobinuria. Br J Haematol. 2011;152(5):631-639.
53. Simak J, Gelderman MP. Cell membrane microparticles in blood and blood products: Potentially pathogenic agents and diagnostic markers. Transfus Med Rev. 2006;20(1):1-26.
54. Kristiansen M, Graversen JH, Jacobsen C, et al. Identification of the haemoglobin scavenger receptor. Nature. 2001;409(6817):198-201.
55. Schaer CA, Vallelian F, Imhof A, Schoedon G, Schaer DJ. CD163-expressing monocytes constitute an endotoxin-sensitive Hb clearance compartment within the vascular system. J Leukoc Biol. 2007;82(1):106-110.
56. Rother RP, Bell L, Hillmen P. The clinical sequelae of intravascular hemolysis a novel mechanism of human disease. JAMA. 2005;293(13):1653-1662.
57. Philippidis P, Mason JC, Evans BJ, et al. Hemoglobin scavenger receptor CD163 mediates interleukin-10 release and heme oxygenase-1 synthesis: antiinflammatory monocyte-macrophage responses in vitro, in resolving skin blisters in vivo, and after cardiopulmonary bypass surgery. Circ Res. 2004;94(1):119-126.
58. Ballarín J, Arce Y, Torra Balcells R, et al. Acute renal failure associated to paroxysmal nocturnal haemoglobinuria leads to intratubular haemosiderin accumulation and CD163 expression. Nephrol Dial Transplant. 2011;26(10):3408-3411.
59. Belcher JD, Chen C, Nguyen J, et al. Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. Blood.123(3):377-390.
60. Olsen SB, Tang DB, Jackson MR, Gomez ER, Ayala B, Alving BM. Enhancement of platelet deposition by cross-linked hemoglobin in a rat carotid endarterectomy model. Circulation. 1996;93(2):327-332.
61. Jacquemin M, Peerlinck K. Free hemoglobin: a boost to platelet thrombi. Blood. 2015;126(20):2261-2262.
62. Studt JD, Kremer Hovinga JA, Antoine G, et al. Fatal congenital thrombotic thrombocytopenic purpura with apparent ADAMTS13 inhibitor: in vitro inhibition of ADAMTS13 activity by hemoglobin. Blood. 2005;105(2): 542-544.
63. Zhou Z, Han H, Cruz MA, López JA, Dong JF, Guchhait P. Haemoglobin blocks von Willebrand factor proteolysis by ADAMTS-13: A mechanism associated with sickle cell disease. Thromb Haemost. 2009;101(6): 1070-1077.
64. Simionatto CS, Cabal R, Jones RL, Galbraith RA. Thrombophlebitis and disturbed hemostasis following administration of intravenous hematin in normal volunteers. Am J Med. 1988;85(4):538-540.
65. Piccin A, Murphy WG, Smith OP. Circulating microparticles: pathophysiology and clinical implications. Blood Rev. 2007;21(3):157-171.
66. van Bijnen STA, van Heerde WL, Muus P. Mechanisms and clinical implications of thrombosis in paroxysmal nocturnal hemoglobinuria. J Thromb Haemost. 2012;10 (1):1-10.
67. Helley D, de Latour RP, Porcher R, et al. Evaluation of hemostasis and endothelial function in patients with paroxysmal nocturnal hemoglobinuria receiving eculizumab. Haematologica. 2010;95(4):574-581.
68. Setty BNY, Betal SG, Zhang J, Stuart MJ. Heme induces endothelial tissue factor expression: potential role in hemostatic activation in patients with hemolytic anemia. J Thromb Haemost. 2008;6(12):2202-2209.
69. Wagener FA, Feldman E, de Witte T, Abraham NG. Heme induces the expression of adhesion molecules ICAM-1, VCAM-1, and E selectin in vascular endothelial cells. Proc Soc Exp Biol Med. 1997;216(3):456-463.
70. Qari MH, Dier U, Mousa SA. Biomarkers of inflammation, growth factor, and coagulation activation in patients with sickle cell disease. Clin Appl Thromb. 2012;18(2):195-200.
71. Maroney SA, Mast AE. Expression of tissue factor pathway inhibitor by endothelial cells and platelets. Transfus Apher Sci. 2008;38:9-14.
72. Broze GJ, Girard TJ, Girard TJ. Tissue factor

pathway inhibitor: structure-function. Front Biosci (Landmark Ed). 2012;17:262-280.
73. Girard TJ, Tuley E, Broze GJ. TFPI Beta is the GPI-anchored TFPI isoform on human endothelial cells and placental microsomes. Blood. 2012;119(5):1256-1262.
74. Maroney SA, Cunningham AC, Ferrel J, et al. A GPI-anchored co-receptor for tissue factor pathway inhibitor controls its intracellular trafficking and cell surface expression. J Thromb Haemost. 2006;4(5):1114-1124.
75. Van Bijnen STA, Osterud B, Barteling W, et al. Alterations in markers of coagulation and fibrinolysis in patients with Paroxysmal Nocturnal Hemoglobinuria before and during treatment with eculizumab. Thromb Res. 2015;136(2):274-281.
76. Weitz JI. Heparan sulfate: Antithrombotic or not? J Clin Invest. 2003;111(7):952-954.
77. HajMohammadi S, Enjyoji K, Princivalle M, et al. Normal levels of anticoagulant heparan sulfate are not essential for normal hemostasis. J Clin Invest. 2003;111(7):989-999.
78. Ferreira V, Pangburn M, Cortés C. Complement control protein factor H: the good, the bad, and the inadequate. Mol Immunol. 2010;47(13):2187-2197.
79. Banerjee D, Mazumder S, Sinha AK. The role of inhibition of nitric oxide synthesis in the aggregation of platelets due to the stimulated production of thromboxaneA2. Blood Coagul Fibrinolysis. 2014;25(6):585-591.
80. Amer J, Zelig O, Fibach E. Oxidative status of red blood cells, neutrophils, and platelets in paroxysmal nocturnal hemoglobinuria. Exp Hematol. 2008;36(4):369-377.
81. Ataga KI, Cappellini MD, Rachmilewitz EA. Beta-thalassaemia and sickle cell anaemia as paradigms of hypercoagulability. Br J Haematol. 2007;139(1):3-13.
82. Iuliano L, Pedersen JZ, Praticò D, Rotilio G, Violi F. Role of hydroxyl radicals in the activation of human platelets. Eur J Biochem. 1994;221(2):695-704.
83. Fuchs TA, Brill A, Duerschmied D, et al. Extracellular DNA traps promote thrombosis. Proc Natl Acad Sci USA. 2010;107(36):15880-15885.
84. Martinod K, Wagner DD. Thrombosis: tangled up in NETs. Blood. 2014;123(18):2768-2776.
85. Nafa K, Bessler M, Mason P, et al. Factor V Leiden mutation investigated by Amplification Created Restriction Enzyme Site (ACRES) in PNH patients with and without thrombosis. Haematologica. 1996;81(6):540-542.
86. Ritis K, Doumas M, Mastellos D, et al. A novel C5a receptor-tissue factor cross-talk in neutrophils links innate immunity to coagulation pathways. J Immunol. 2006;177(7):4794-4802.
87. von Vietinghoff S, Tunnemann G, Eulenberg C, et al. NB1 mediates surface expression of the ANCA antigen proteinase 3 on human neutrophils. Blood. 2007;109(10):4487-4493.
88. Renesto P, Si-Tahar M, Moniatte M, et al. Specific inhibition of thrombin-induced cell activation by the neutrophil proteinases elastase, cathepsin G, and proteinase 3: evidence for distinct cleavage sites within the aminoterminal domain of the thrombin receptor. Blood. 1997;89(6):1944-1953.
89. Villegas-Mendez A, Montes R, Ambrose LR, Warrens AN, Laffan M, Lane DA. Proteolysis of the endothelial cell protein C receptor by neutrophil proteinase 3. J Thromb Haemost. 2007;5(5):980-988.
90. Steppich BA, Seitz I, Busch G, Stein A, Ott I. Modulation of tissue factor and tissue factor pathway inhibitor-1 by neutrophil proteases. Thromb Haemost. 2008;100(6):1068–1075.
91. Huber-Lang M, Sarma JV, Zetoune FS, et al. Generation of C5a in the absence of C3: a new complement activation pathway. Nat Med. 2006;12(6):682-687.
92. Leung LL, Morser J. Plasmin as a complement C5 convertase. EBioMedicine. 2016;5:20-21.
93. Foley JH, Walton BL, Aleman MM, et al. Complement activation in arterial and venous thrombosis is mediated by plasmin. EBioMedicine. 2016;5:175-182.
94. Montrucchio G, Bergerone S, Bussolino F, et al. Streptokinase induces intravascular release of platelet-activating factor in patients with acute myocardial infarction and stimulates its synthesis by cultured human endothelial cells. Circulation. 1993;88:1476-1483.
95. Lupia E, Del Sorbo L, Bergerone S, Emanuelli G, Camussi G, Montrucchio G. The membrane attack complex of complement contributes to plasmin-induced synthesis of platelet-activating factor by endothelial cells and neutrophils. Immunology. 2003;109(4):557-563.
96. Liu X, Miller MJS, Joshi MS, Sadowska-Krowicka H, Clark DA, Lancaster JR. Diffusion-limited reaction of nitric oxide with erythrocytes. J Biol Chem. 1998;273(30):18709-18713.
97. Schnog JJB, Jager EH, van der Dijs FPL, et al. Evidence for a metabolic shift of arginine metabolism in sickle cell disease. Ann Hematol. 2004;83(6):371-375.
98. Hill A, Sapsford RJ, Scally A, et al. Under-recognized complications in patients with paroxysmal nocturnal haemoglobinuria: Raised pulmonary pressure and reduced right ventricular function. Br J Haematol. 2012;158(3):409-414.
99. Freedman JE, Loscalzo J. Nitric oxide and its relationship to thrombotic disorders. J Thromb Haemost. 2003;1(6):1183-1188.
100. Morel O, Jesel L, Freyssinet JM, Toti F. Cellular mechanisms underlying the formation of circulating microparticles. Arterioscler Thromb Vasc Biol. 2011;31(1):15-26.
101. Bridge KI, Philippou H, Ariëns R. Clot properties and cardiovascular disease. Thromb Haemost. 2014;112(5):901-908.
102. Collet JP, Park D, Lesty C, et al. Influence of fibrin network conformation and fibrin fiber diameter on fibrinolysis speed: dynamic and structural approaches by confocal microscopy. Arterioscler Thromb Vasc Biol. 2000;20(5):1354-1361.
103. Ajjan R, Lim BC, Standeven KF, et al. Common variation in the C-terminal region of the fibrinogen β-chain: effects on fibrin structure, fibrinolysis and clot rigidity. Blood. 2008;111(2):643-650.
104. Shats-Tseytlina EA, Nair CH, Dhall DP. Complement activation: a new participant in the modulation of fibrin gel characteristics and the progression of atherosclerosis? Blood Coagul Fibrinolysis. 1994;5(4):529-535.
105. Howes JM, Richardson VR, Smith KA, et al. Complement C3 is a novel plasma clot component with anti-fibrinolytic properties. Diabetes Vasc Dis Res. 2012;9(3):216-25.
106. Hess K, Ajjan R, Phoenix F, Dobó J, Gál P, Schroeder V. Effects of MASP-1 of the complement system on activation of coagulation factors and plasma clot formation. PLoS One. 2012;7(4):e35690.
107. Undas A. Fibrin clot properties and their modulation in thrombotic disorders. Thromb Haemost. 2014;112(1):32-42.
108. Wolberg AS, Monroe DM, Roberts HR, Hoffman M. Elevated prothrombin results in clots with an altered fiber structure: A possible mechanism of the increased thrombotic risk. Blood. 2003;101(8):3008-3013.
109. Lipinski B, Pretorius E, Oberholzer HM, van der Spuy WJ. Interaction of fibrin with red blood cells: the role of iron. Ultrastruct Pathol. 2012;36(2):79-84.
110. Martinez M, Weisel JW, Ischiropoulos H. Functional impact of oxidative post-translational modifications on fibrinogen and fibrin clots. Biophys Chem. 2005;257(5):2432-2437.
111. Aleman MM, Gardiner C, Harrison P, Wolberg AS. Differential contributions of monocyte- and platelet-derived microparticles towards thrombin generation and fibrin formation and stability. J Thromb Haemost. 2011;9(11):2251-2261.
112. Ploug M, Plesner T, Ebbe R, et al. The receptor for urokinase-type plasminogen activator is deficient on peripheral blood leukocytes in patients with paroxysmal nocturnal hemoglobinuria. Blood. 1992;79(1):1447-1455.
113. Ninomiya H, Hasegawa Y, Nagasawa T, Abe T. Excess soluble urokinase-type plasminogen activator receptor in the plasma of patients with paroxysmal nocturnal hemoglobinuria inhibits cell-associated fibrinolytic activity. Int J Hematol. 1997;65(3):285-291.
114. Rønne E, Pappot H, Grøndahl-Hansen J, et al. The receptor for urokinase plasminogen activator is present in plasma from healthy donors and elevated in patients with paroxysmal nocturnal haemoglobinuria. Br J Haematol. 1995;89(3):576-581.
115. Rendu F, Brohard-Bohn B. The platelet release reaction: granules' constituents, secretion and functions. Platelets. 2001;12(5):261-273.
116. Baalasubramanian S, Harris CL, Donev RM, et al. CD59a Is the primary regulator of membrane attack complex assembly in the mouse. J Immunol. 2004;173(6):3684-3692.
117. Song WC, Deng C, Raszmann K, et al. Mouse decay-accelerating factor: selective and tissue-specific induction by estrogen of the gene encoding the glycosylphosphatidylinositol-anchored form. J Immunol. 1996;157(9):4166-4172.
118. Xu C, Mao D, Holers VM, Palanca B, Cheng AM, Molina H. A critical role for murine complement regulator crry in fetomaternal tolerance. Science. 2000;287(5452):498-501.
119. Kim DD, Miwa T, Song WC. Retrovirus-mediated over-expression of decay-accelerating factor rescues Crry-deficient erythrocytes from acute alternative pathway complement attack. J Immunol. 2006;177(8):5558-5566.
120. Rosti V, Tremml G, Soares V, Pandolfi PP, Luzzatto L, Bessler M. Murine embryonic stem cells without pig-a gene activity are competent for hematopoiesis with the PNH phenotype but not for clonal expansion. J Clin Invest. 1997;100(5):1028-1036.
121. Tremml G, Dominguez C, Rosti V, et al. Increased sensitivity to complement and a decreased red blood cell life span in mice mosaic for a nonfunctional Piga gene. Blood. 1999;94(9):2945-2954.