Thrombotic complications in paroxysmal nocturnal haemoglobinuria: a literature review

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Introduction

Paroxysmal nocturnal haemoglobinuria (PNH) is a rare, clonal haematopoietic stem cell disease caused by mutations in an X-linked gene called phosphatidylinositol glycan class A (*PIG-A*). The disease can present with bone marrow failure, haemolytic anaemia, smooth muscle dystonia and thrombosis. The *PIG-A* gene product is necessary for the first step in the biosynthesis of glycosylphosphatidyinositol (GPI) anchors - the transfer of GlcNAc from UDP-GlcNAc to form *N*-acetylglucosaminyl phosphatidylinositol (GlcNAc-PI) - where expansion and differentiation of the PIG-A mutant stem cell leads to clinical manifestations of the disease[1].

PNH may occur *de novo* (classical PNH) or in the setting of aplastic anaemia (hypoplastic PNH); the absence of GPI-linked complement regulatory proteins on PNH erythrocytes renders these cells susceptible to terminal complement-mediated haemolysis[2]. The natural history of PNH is highly variable, ranging from indolent to life-threatening. The median survival is 10 to 15 years, but the range is wide. Thrombosis is the leading cause of death, and an initial thrombotic event increases the relative risk of death by 5- to 10-fold in PNH[3]. Haemolysis most likely contributes to thromboembolism, as patients with larger PNH clones have a higher incidence of thromboembolism and events have been temporally associated with increased haemolysis. Although the mechanism is not fully understood, haemolysis has been implicated in the initiation of platelet activation and aggregation[4].

This review discusses the underlying mechanisms that lead to thrombosis in PNH and therapeutic approaches.

Underlying mechanisms of thrombosis

Paroxysmal nocturnal hemoglobinuria is associated with an increased risk of thrombosis through unknown processes[5-7]. Possible mechanisms include: procoagulant microparticles released by complement-mediated platelet activation; chronic hypofibrinolysis through altered plasminogen activation, possibly due to a decrease in urinary plasminogen activator receptor expression on leucocyte surfaces; release of free haemoglobin by chronic haemolysis, leading to nitric oxide depletion and, subsequently, endothelial dysfunction and platelet activation[8,10].

In response to complement injury, the red blood cell membrane is disrupted in PNH, resulting in increased circulating microvesicles[11]. Membrane vesiculation results in the exposure of phosphatidyl serine from the inner membrane leaflet, an event which has been linked to thrombosis[12]. It has been proposed that the phosphatidyl serine provides the negatively-charged surface needed for the assembly of the prothrombinase complex[13]. Endothelial cell vesiculation has been reported and endothelial cell activation along with increased expression of von Willebrand factor has been noted in PNH[14].

Urokinase-type plasminogen activator is one of two serine proteases involved in extracellular proteolysis, through the ability to convert plasminogen into plasmin[15]. Besides the well-established regulation of fibrinolysis during thrombosis, plasmin also plays a role in the degradation of extracellular matrix and basement membrane proteins during tissue remodelling and cell migration. A very specific cellular receptor, binding urokinase-type plasminogen activator with a high affinity (kd −0.1 to 1 nmol/L), may provide cells with a mechanism for localised plasmin formation in close proximity to the plasma membrane. The absence of this urokinase-type plasminogen activator receptor from the surface of PNH-affected leucocytes and the consequently reduced potential of these cells to generate plasmin may, therefore, have a significant contribution to the pathogenesis of this disease[16,17].

*Blood Transfus 2012; **10**: 428-35* DOI 10.2450/2012.0161-11

Because haemolysis in PNH is intravascular, nitric oxide scavenging occurs due to the presence of free haemoglobin in plasma. Nitric oxide consumption is 6- to 10-fold higher in patients with haemolytic PNH than in patients with another form of haemolytic anaemia[18] Nitric oxide depletion may contribute to the development of arterial constriction with reduced blood flow to the kidneys as well as to both systemic and pulmonary arterial hypertension[19]. It is also interesting that, although often attributed to intravascular haemolysis, thrombosis may occur in patients with minimal or no evidence of haemolysis. Thrombosis has been reported in patients without overt evidence of haemolysis, with smaller clones, mild anaemia and no transfusions.

Retrospective studies have suggested that the risk of thrombosis might be correlated with the size of the PNH granulocyte clone[20]. Hall *et al.*[21] addressed the risk factors for thrombosis and the role of warfarin prophylaxis in PNH. The overall cumulative incidence (at 20 years) of thrombosis in patients with granulocyte clone sizes larger than 50%, excluding those taking warfarin as primary thromboprophylaxis, was 53.5%. Granulocyte clone sizes larger than 50% were found to be highly predictive of thrombotic risk, with an incidence of thrombosis of only 5.8% in patients with small PNH granulocyte clones. A cut-off was taken at 50% as this provided the best delineation between aplastic and haemolytic PNH in this group of patients.

The risk of thromboembolic events increases by 1.64-fold for every 10% increase in the size of the white blood cell clone (granulocytes/monocytes). Activation of granulocytes leads to the release of inflammatory molecules that damage the endothelium. Complement damaged leucocytes adhere to endothelial cells and may induce tissue factor expression, thereby promoting the release of inflammatory cytokines[20,21]. Complement protein C5a, a potent inflammatory protein, binds to a receptor on granulocytes, thus accelerating the recruitment and activation of granulocytes and monocytes and is considered to be a possible link between inflammation and thrombosis[22]. The lack of the complement regulatory proteins CD55 and CD59 on the surface of PNH platelets, which renders these cells more sensitive to complement-mediated activation, may also contribute to the thrombotic tendency in patients with PNH[23].

Even though the hypercoagulable state is responsible for life-threatening venous thrombosis in patients with PNH, the evaluation of acquired and inherited thrombophilic factors for thromboembolism have so far been only partially investigated. Dragoni *et al.*[24] investigated thrombophilia in patients affected by PNH who were diagnosed by the HAM test and flow cytometric studies. All tests were performed at least 6 months after the thrombotic event. Out of 13 PNH patients, five (38.4%) had experienced a thrombotic event whereas no episodes occurred in healthy subjects ($P<0.0001$) or in patients with aplastic anaemia ($P=0.039$). Antithrombin, protein C and protein S were normal in all patients with PNH and aplastic anaemia, as well as in healthy subjects. No differences were found regarding the prevalence of FV R506Q, FII (G20210A) and the thermolabile variant C677T of MTHFR between PNH patients and healthy subjects. Homocysteine levels were also normal in all PNH patients. Pathological levels of anti-phospholipid antibodies (APA) were found in eight PNH patients (61.5%), five of whom had a history of thrombosis. The authors hypothesised that immune-system involvement in patients with PNH could contribute to the development of APA. However, these data were not confirmed by others; Darnige *et al*.[28] examined the possible impact of eculizumab on levels of APA (lupus anticoagulant, anti-β2GPI and anticardiolipin) in PNH patients. They found a lower prevalence of APA (3/20, 15%) than Dragoni *et al.* (2010), despite using the same positivity cut-off (99th percentile). In addition, none of their patients had high APA levels, none of their patients who had thrombotic events exhibited APA and they found no significant difference between APA levels before and 11 weeks after eculizumab administration. Thus, the authors concluded that the effect of eculizumab on thrombosis does not seem to be related to a reduction in microparticle or APA levels.

Shamseddine *et al.*[25] reported the first PNH patient who concomitantly had two inherited thrombophilia mutations, being heterozygous for both FII (G20210A) and MTHFR C677T mutations. The patient had bilateral deep vein thrombosis (DVT) and was maintained on oral anticoagulation therapy. Despite this, he developed hepatic vein thrombosis.

The JAK2-V617F mutation is frequently found in myeloproliferative neoplasms. Like PNH,

myeloproliferative neoplasma are associated with a high risk of thrombosis at similar locations, raising the question of whether the JAK2-V617F mutation is involved in PNH as well. No JAK-V617F mutations were found by Fouassier *et al*.[26] in 11 PNH patients with varying clone sizes (range: 0.5-92%), including three patients with thrombosis, whereas Sugimori *et al*.[27] reported that, among 21 PNH patients with Budd-Chiari syndrome, three patients with classic PNH had JAK-V617F mutations.

Eculizumab is a humanised monoclonal antibody that binds to complement protein 5 (C5), thereby inhibiting the formation of the terminal components of the complement cascade (also called the membrane attack complex or MAC) during complement activation. Its use has been associated with a reduction in haemolysis, stabilisation of haemoglobin levels, reduction in transfusion requirements and improvement in quality of life. Eculizumab also protects against the complications of haemolytic PNH, such as deteriorating renal function, pulmonary hypertension and thromboembolism[28].

Several reports suggest that eculizumab plays a crucial role in inducing a significant and sustained decrease in the activation of haemostasis, and probably contributes to the protective effect on thrombosis in PNH patients, for which there is both laboratory and clinical evidence (Table I).

Helley *et al*.[29] found that eculizumab significantly reduced the plasma levels of prothrombin fragment F1+2 (F1+2), D-dimers and plasmin-antiplasmin complexes during the induction phase (week 5) and during chronic treatment (week 11), and even at week 47, in the nine patients tested. These authors studied 23 patients with PNH, before and after 5 and 11 weeks of treatment with eculizumab. They examined markers of thrombin generation, reactional fibrinolysis and endothelial dysfunction: F1+2, D-dimers, plasmin-antiplasmin complexes, tissue plasminogen activator, plasminogen activator inhibitor, soluble thrombomodulin, intercellular adhesion molecule 1, vascular cell adhesion molecule 1, endothelial microparticles and tissue factor pathway inhibitor. At baseline, von Willebrand factor, soluble vascular cell adhesion molecule 1, endothelial microparticle count and F1+2 and D-dimer levels were significantly elevated in patients, including those with no history of clinical thrombosis. These results indicate a decrease in coagulation activation and fibrinolysis during eculizumab therapy. Patients receiving anticoagulant treatment after a thrombotic event had even lower F1+2 plasma levels, which were probably related to the anticoagulant treatment itself.

Treatment with eculizumab was associated with significant decreases in plasma markers of coagulation activation (F1+2, P=0.012, and D-dimers, P=0.01), and reactional fibrinolysis (plasma-antiplasmin complexes, P=0.0002). Eculizumab treatment also significantly reduced plasma markers of endothelial cell activation (tissue plasminogen activator, P=0.0005, soluble vascular cell adhesion molecule 1, P<0.0001, and von Willebrand factor, P=0.0047) as well as total (P=0.0008) and free (P=0.0013) tissue factor pathway inhibitor plasma levels. The authors concluded that the terminal complement inhibitor, eculizumab, induced a significant and sustained decrease in the activation of both the plasma haemostatic system and the vascular endothelium, probably contributing to the protective effect of eculizumab on thrombosis in this setting.

Weitz *et al*.[30] hypothesised that the reduction in the rate of thromboembolic events during treatment with eculizumab is due to a decrease in leucocyte complement injury with a reduction in leucocyte-derived tissue factor microparticles, a decrease in systemic thrombin generation and a reduction in inflammatory cytokines. Markers of haemostatic

Table I - Thrombosis rates with/without oral anticoagulation therapy and with/without eculizumab treatment.

Study	Sample size	DVT rate with OAT	DVT rate with eculizumab	DVT rate without OAT	DVT rate without eculizumab	P value
Hall (2003)	163 PNH	0% (36 pts)	NA	36.5% (56 pts)	NA	(P=0.01)
Hillmen (2007)	195 PNH	0.62 per 100 pts/y	1.07 per 100 pts/y (3 events)	10.61 per 100 pts/y	7.37 per 100 pts/y (124 events)	(P=0.001)
Kelly (2011)	79 (PNH)	1 event	0.8 per 100 pts/y (2 events)	17 events (74%)	5.6 per 100 pts/y (34 events)	(P=0.001)

Legend: OAT: oral anticoagulant therapy; DVT: deep vein thrombosis; PNH: paroxysmal nocturnal haemoglobinuria.

activation, D-dimers and thrombin-antithrombin complexes were elevated in all but two of the 11 patients prior to beginning eculizumab treatment. There was a highly significant decrease in markers of haemostatic activation (thrombin-antithrombin complexes, D-dimers) as well as markders of inflammation markers (interleukin-6) during the 4 weeks of induction therapy, and levels remained low over the 90 days of the study.

Recently, Kelly *et al.*[31] evaluated 79 consecutive patients treated with eculizumab. The survival of patients treated with eculizumab was not different to that of an age- and sex- matched normal control population (P=0.46), but was significantly better than that of 30 similar patients with PNH managed in the 7 years before eculizumab (P=0.030). Three patients on eculizumab, all over 50 years old, died from causes unrelated to PNH. Twenty-one of the 79 patients (27%) had a thrombosis prior to starting eculizumab (5.6 events per 100 patient years) compared with two thrombotic episodes on eculizumab (0.8 events per 100 patient years; P<0.001). Twenty-one patients with no prior history of thrombosis discontinued warfarin after starting eculizumab with no thrombotic sequelae. Forty of 61 (66%) patients on treatment with eculizumab for over 12 months achieved transfusion independence. In this series, the authors found both a significantly worse survival in patients with PNH in the 7 years immediately prior to eculizumab (P=0.030), and no difference in mortality between patients on eculizumab and the normal population (P=0.46).

Interestingly, in another analysis, Hillmen *et al.*[32] evaluated whether long-term treatment with the complement inhibitor eculizumab reduces the rate of thromboembolism in patients with PNH. They conducted a prospective analysis of patients' data pooled from three clinical studies comparing the rates of thromboembolic events in untreated and in eculizumab-treated patients with PNH. This analysis demonstrated that eculizumab treatment resulted in a dramatic reduction in thromboembolism from 7.37 to 1.07 events per 100 patient-years. With equalisation of the duration of exposure before and during treatment for each patient, there was a reduction from 39 events before eculizumab to three events during eculizumab (P<0.001). The thromboembolic event rate decreased from 10.61 to 0.62 events per 100 patient-years with eculizumab treatment (P<0.001).

Although the rate of pre- and post-eculizumab thrombosis was evaluated in the PNH population, no trial prospectively compared the rate of thrombosis between eculizumab-treated and untreated patients[29-31].

Incidence of thrombosis in paroxysmal nocturnal haemoglobinuria

PNH is associated with a markedly-increased risk of venous thrombosis that can occur in both common sites (limbs and lungs) and uncommon ones such as the hepatic veins (Budd-Chiari syndrome), the cavernous sinus and the mesenteric veins[32]. Venous thromboembolism in critical anatomic sites (cerebral and splanchnic circulation) is a major cause of morbidity and mortality in patients with PNH. Once an initial episode of thrombosis occurs, the risk of further thrombotic events is much higher and carries a 5- to 10-fold increase in mortality.

Ziakas *et al.*[33] conducted an extensive review on the sites of thrombosis, risks and outcomes in patients with PNH. The analysis showed that 25% of thrombotic events were fatal with 20.5% involving more than one site. Age at the time of thrombosis was an independent predictor of death (RR, 1.04; 95% CI: 1.01-1.07, per 1-year increase). Hepatic vein thrombosis leading to Budd-Chiari syndrome was the most frequent (40.7%) thrombotic complication of PNH. Anticoagulation alone is unlikely to restore previous hepatic flow, and thus invasive interventions have been proposed, such as angioplasty of the inferior vena cava and hepatic veins, transjugular intrahepatic portosystemic shunts and surgical approaches including porto-caval, spleno-renal and meso-caval shunts. Cerebral vein and sinus thrombosis was the second most common type of thrombosis in the above cohort, with the superior sagittal sinus being the most frequently involved site. Pulmonary embolism was not associated with DVT and in a significant number of affected individuals it was detected coincidentally during autopsy. Pregnancy should not be recommended in patients suffering from PNH; 11 thrombotic episodes were identified during pregnancy and the postpartum period. These episodes involved the hepatic veins, central nervous system arteries and veins, cerebral sinus, pulmonary vasculature, portal vein,

inferior vena cava and deep veins. Three fatalities occurred. In a site-adjusted multivariate analysis, hepatic vein thrombosis (RR, 5.64; 95% CI: 2.26-14.02), pulmonary embolism (RR, 5.70; 95% CI: 1.70-19.11), mesenteric vein thrombosis (RR, 8.27; 95% CI: 2.50-27.41), and venous stroke (RR, 4.87; 95% CI: 1.79-13.30) were significant predictors of thrombosis-related mortality.

The risk of venous thrombosis is correlated with PNH granulocyte clone size. Moyo *et al*.[34] confirmed the association between PNH granulocyte clone size and thrombosis, and estimated an odds ratio of 1.64 for every 10% increase in clone size. Whether PNH clone size in other lineages also correlates with thrombotic risk is unknown. However, this can be expected for PNH platelet clone size, since this correlates strongly with PNH granulocyte clone size.

Geographic differences in the incidence of thrombosis in PNH have been reported. Araten *et al*.[35] reviewed 64 patients with "classic PNH" from a single institution to determine the rate of thrombosis in different ethnic groups. When they compared African-Americans[36] (n=11) and Latin-Americans (n=8) with other patients (n=45), it was found that these former groups are at increased risk of thrombosis [hazard ratio 3.66 (P=0.005) and 3.52 (P=0.035), respectively, by Cox regression analysis]. Their data also suggest that this difference in the rate of thrombosis has an impact on survival. A lower risk was reported in Chinese and Japanese patients, which can probably be explained by a significantly lower PNH granulocyte size in these patients than in Western patients[37]. These findings indicate that ethnicity is a risk factor for thrombosis in PNH, and have implications for decision-making regarding the management of these patients, including the prevention of thrombosis.

The role of antithrombotic therapy

Thrombosis is the leading cause of death from PNH and should be treated promptly with anticoagulation and/or thrombolytic therapy depending on the site of the thrombus. However, anticoagulation is only partially effective at preventing thrombosis in PNH[38]. Although great progress has been made, some important questions, discussed below, remain unanswered.

How should a PNH patient with risk factors additional to the PNH clone be managed?

Hall *et al*.[38] conducted a study to identify factors predictive of thrombosis and to investigate the role of primary prophylaxis with warfarin in order to prevent thrombosis in those patients found to be at a particularly higher risk of thrombosis. In this unselected group of 163 patients followed over a median of 6 years, the rate of venous thrombosis was comparable to that in previous studies[5]. The overall cumulative incidence (at 20 years) of thrombosis in patients with granulocyte clone sizes larger than 50%, excluding those taking warfarin as primary thromboprophylaxis, was 53.5%. Patients with PNH granulocytes greater than 50% (including those on primary warfarin prophylaxis) were found to have a 10-year cumulative incidence rate of thrombosis of 34.5% whereas those with clone sizes smaller than 50% had a thrombosis rate of 5.3% (P=0.01). When patients on primary thromboprophylaxis were excluded, the 10-year cumulative incidence rate of thrombosis in those with large clones was 44%, compared with 5.8% in those with clone sizes smaller than 50% (P=0.01). In the group of 39 patients on primary thromboprophylaxis, there were two warfarin-associated haemorrhages (one of which led to the patient's death) with a major bleeding risk higher than that reported in oral anticoagulation clinics (Palareti *et al*.[32] found the risk of haemorrhagic events was 1.1 major bleeding events per 100 patient-years and 0.25 fatalities per 100 patient-years). Nevertheless, this study was the basis for the recommendation of the international PNH interest group to consider vitamin K antagonist prophylaxis in patients with a PNH granulocyte clone greater than 50% and no contraindications to such prophylaxis.

The optimal therapeutic range for the International Normalised Ratio has yet to be assessed but the target in this series of patients was between 2.0 and 3.0, which appears to prevent the occurrence of thrombosis. However, the risk reduction provided by vitamin K antagonism in PNH patients was lower than expected in the warfarin-treated population at higher risk of thrombosis[32]. The authors concluded that primary prophylaxis with warfarin should be considered in PNH patients if the granulocyte clone size is larger than 50%, the platelet count is stable

at greater than $100x10^9$/L and there are no known contraindications to anticoagulation.

Thrombolytic therapy with tissue plasminogen activator may be life-saving in some cases; indeed, there have been various reports on the efficacy of the systemic tissue plasminogen activator administration in reversing one of the most serious complications of PNH, namely the Budd-Chiari syndrome[39,40]. Recently, Araten *et al*.[41] described nine patients in whom this treatment resulted in resolution of the thrombus. However, in one patient, bleeding may have contributed to the patient's death.

What is the role of long-term, primary antithrombotic prophylaxis in patients untreated with eculizimab?

It is controversial whether PNH patients not taking eculizumab should receive prophylactic anticoagulation and whether patients on eculizumab therapy, who have had a prior thrombus, should remain on anticoagulation[42]. Prophylactic anticoagulation has not been demonstrated to prevent thrombosis in PNH patients and may even be dangerous since low platelet counts are often observed in people with PNH. Thus, in patients who are not candidates for eculizumab therapy, initiation of prophylactic anticoagulation it is not recommended. Possible exceptions include patients with persistently-elevated D-dimer levels, pregnant PNH patients and patients in the perioperative period. Discontinuing anticoagulation in patients on eculizumab with a history of previous thrombosis is even more controversial, and there are insufficient data to make any recommendations[43].

Should all PNH patients receive long-term vitamin K antagonist therapy after a single thrombotic episode?

Many results suggest that chronic administration of eculizumab can significantly reduce the overall life-time thromboembolic event rate[44]. Nevertheless, a major unresolved issue in the management of PNH patients on eculizumab is whether or not anticoagulation can be safely discontinued, especially in patients with previous thrombotic events. Recent phase III studies of eculizumab in PNH patients demonstrate that that this monoclonal antibody markedly reduces the risk of thrombosis and that prophylactic anticoagulation in PNH patients does little to reduce the risk of thrombosis in PNH patients not on eculizumab[44]. Specifically, the thromboembolism event rate per 100 patient-years following eculizumab administration was reduced from 7.4 events to 1.1 events, (RRR of 85%). In antithrombotic-treated patients, the thromboembolism event rate was also reduced (10.6 events per 100 patient-years to 0.6 events per 100 patient-years) with eculizumab treatment. Interestingly, the use of antithrombotic therapy before eculizumab did not appear to influence the risk of thrombosis (7.4 events per 100 patient-years versus 10.6 events per 100 patient-years); furthermore, eculizumab markedly reduced the risk of thrombosis regardless of whether patients were or were not receiving anticoagulation. Thus, a question of major clinical importance is whether life-long anticoagulation is necessary for PNH patients who are well controlled on eculizumab therapy. Emadi *et al*.[45] suggested that it may be safe to withdraw anticoagulation in PNH patients on eculizumab. The decision of whether or not it is safe to withdraw anticoagulation in PNH patients on eculizumab should actually be determined by the results of a randomised controlled clinical trial, but given the rarity of PNH, it is unlikely that such a trial will ever be performed.

Keywords: paroxysmal nocturnal haemoglobinuria (PNH), thrombosis.

The Authors declare no conflicts of interest.

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Arrived: 28 November 2011 - Revision accepted: 23 March 2012
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