





## RESEARCH ARTICLE

# Patients with paroxysmal nocturnal hemoglobinuria demonstrate a prothrombotic clotting phenotype which is improved by complement inhibition with eculizumab

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## Abstract

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare hematological disorder, characterized by complement-mediated intravascular hemolysis and thrombosis. The increased incidence of PNH-driven thrombosis is still poorly understood, but unlike other thrombotic disorders, is thought to largely occur through complement-mediated mechanisms. Treatment with a C5 inhibitor, eculizumab, has been shown to significantly reduce the number of thromboembolic events in these patients. Based on previously described links between changes in fibrin clot structure and thrombosis in other disorders, our aim was to investigate clot structure as a possible mechanism of thrombosis in patients with PNH and the anti-thrombotic effects of eculizumab treatment on clot structure. Clot structure, fibrinogen levels and thrombin generation were examined in plasma samples from 82 patients from the National PNH Service in Leeds, UK. Untreated PNH patients were found to have increased levels of fibrinogen and thrombin generation, with subsequent prothrombotic changes in clot structure. No link was found between increasing disease severity and fibrinogen levels, thrombin generation, clot formation or structure. However, eculizumab treated patients showed decreased fibrinogen levels, thrombin generation and clot density, with increasing time spent on treatment augmenting these anti-thrombotic effects. These data suggest that PNH patients have a prothrombotic clot phenotype due to increased fibrinogen levels and thrombin generation, and that the antithrombotic effects of eculizumab are, in-part, due to reductions in fibrinogen and thrombin generation with downstream effects on clot structure.

## 1 | INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare life-threatening disorder of hematopoietic stem cells, estimated at 0.1-0.2/100 000 persons per year, characterized by increased sensitivity to complement

attack with or without intravascular hemolysis.<sup>1</sup> Thrombosis is the most serious complication, and accounts for 40%-67% of deaths in PNH.<sup>2</sup> An acquired mutation in the X-linked phosphatidylinositol glycan class A gene (*PIG-A*) results in the loss of glycosylphosphatidyinositol (GPI) anchors leading to mature blood cells lacking essential surface proteins.

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This includes complement regulatory proteins CD55 and CD59.<sup>3</sup> CD55 inhibits C3 convertase formation, and CD59 regulates membrane attack complex (MAC) formation, reducing pore formation and cell lysis.<sup>4</sup> Without these regulatory proteins, complement attack is thought to result in erythrocyte lysis, platelet activation and loss of thrombotic modulators on granulocytes, causing many PNH symptoms.<sup>5,6,7</sup> Disease severity is dependent on the number of PNH affected blood cells, which can range from 0.02%-100%. Moyo et al. showed correlation between increasing PNH cell proportion and thrombosis risk, with an odds ratio for thrombosis of 1.64 for each 10% increase in PNH affected cells.<sup>8</sup>

The mechanisms involved in PNH-driven thrombosis are poorly understood, despite the discovery of eculizumab, a humanized monoclonal antibody directed against complement C5, which drastically reduces thromboembolic events in PNH patients.<sup>9,10</sup> Proposed mechanisms suggest a complement-mediated prothrombotic state in conjunction with platelet abnormalities and impaired fibrinolysis.<sup>11,12</sup> Another as yet unexplored mechanism involves possible changes in fibrin clot structure. Many previous studies have demonstrated links between altered clot structure and thrombosis in other diseases.<sup>13</sup> In view of this, clot structure was examined in 82 PNH patients to determine if alterations in clot structure may contribute to increased thrombosis risk in PNH, and to explore if treatment with eculizumab normalizes such alterations.

## 2 | METHODS

### 2.1 | Patients and bloods

Patients (104) from the PNH National Service Clinic, Leeds NHS Teaching Hospitals were enrolled. Participation was voluntary with patients recruited during routine clinical appointments. Twenty-two patients were removed from the analysis due to current anticoagulant therapy with heparin or oral anticoagulants. Twenty medication-free, apparently healthy volunteers with no history of thrombosis, composed of 10 males and 10 females, were recruited as a control group to provide clotting parameters representative of a healthy population. Free flowing venous blood was collected from the antecubital vein on 0.109 M sodium citrate and centrifuged at 2400g for 20 minutes to prepare platelet-poor plasma, which was frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Ethical approval was obtained from the Leeds East Research Ethics Committee (REC reference 16/YH/0290). Written informed consent was received from each patient and volunteer prior to inclusion in the study in accordance with the declaration of Helsinki.

### 2.2 | PNH diagnosis

The PNH diagnosis was confirmed by determination of GPI-deficient cells using flow cytometry as described.<sup>14</sup> Percentage of PNH granulocytes was calculated and used as an indicator of disease severity as these are not affected by hemolysis or transfusions. Serum lactate

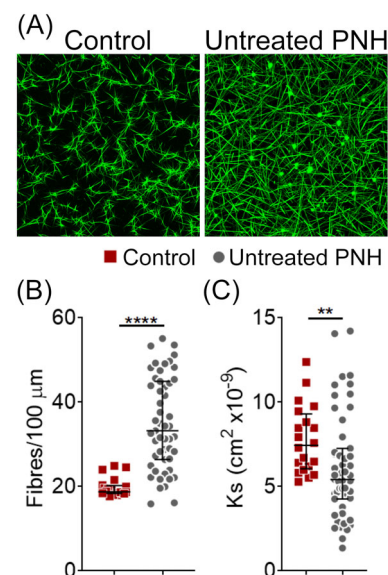
dehydrogenase (LDH) levels were measured as a marker of intravascular hemolysis using a Bayer Advia 1650 or 2400 (Siemens Healthcare, Surrey, UK). The adult reference range for LDH is 160-430 IU/L.

### 2.3 | Clot permeability

Permeability of plasma clots from PNH patients was analyzed by adding 10  $\mu\text{L}$  of activation mixture (human thrombin (0.1 U/mL, Merck Millipore, Watford, UK) and  $\text{CaCl}_2$  (10 mM) final concentrations) to 100  $\mu\text{L}$  of plasma as described.<sup>15</sup> Clot permeability was determined in triplicate by calculating Darcy's constant (Ks), which is a measure of the average pore size of the fibrin network.<sup>16</sup>

### 2.4 | Confocal microscopy

Laser scanning confocal microscopy (LSCM) was performed on plasma clots to analyze fibrin network structure. Plasma samples were diluted by half and spiked with 25  $\mu\text{g}/\text{mL}$  of AlexaFluor488-labeled fibrinogen. Clotting was initiated with 10 mM  $\text{CaCl}_2$  and 0.1 U/mL thrombin. Immediately after clotting initiation, 30  $\mu\text{L}$  was transferred to a channel of an uncoated  $\mu$ -slide VI 0.4 Ibidi slide (Thistle Scientific, UK). The samples were stored in dark humidity chambers for at least 4 hours. Slides were imaged using an inverted Zeiss LSM880 microscope (Carl Zeiss; Welwyn Garden City, UK) with a 40 $\times$  oil immersion objective lens. Fibrin clots were prepared in duplicate and six images were taken per clot. The



**FIGURE 1** The effect of PNH on clot structure. A, Representative confocal microscopy images (Zeiss LSM880,  $\times 40$  oil immersion, NA 1.4,  $200 \mu\text{m} \times 200 \mu\text{m}$ ) of plasma clots from healthy controls and PNH patients not on treatment. B, Effect of PNH on clot density (fibers/100  $\mu\text{m}$ ). C, Effect of PNH on clot porosity (Ks,  $\text{cm}^2 \times 10^{-9}$ ). Dunn's multiple comparisons test, Error bars - median and IQR. Ecu - eculizumab.  $**P \leq .01$ ,  $****P \leq .0001$  [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

Z-stacks (20  $\mu\text{m}$ , 30 slices) were combined to form 3D images (ZEN 2.1 black, Carl Zeiss, Cambridge, UK). Fiber density was calculated as fibers/100  $\mu\text{m}$  using an in-house macro.

## 2.5 | Turbidity and lysis

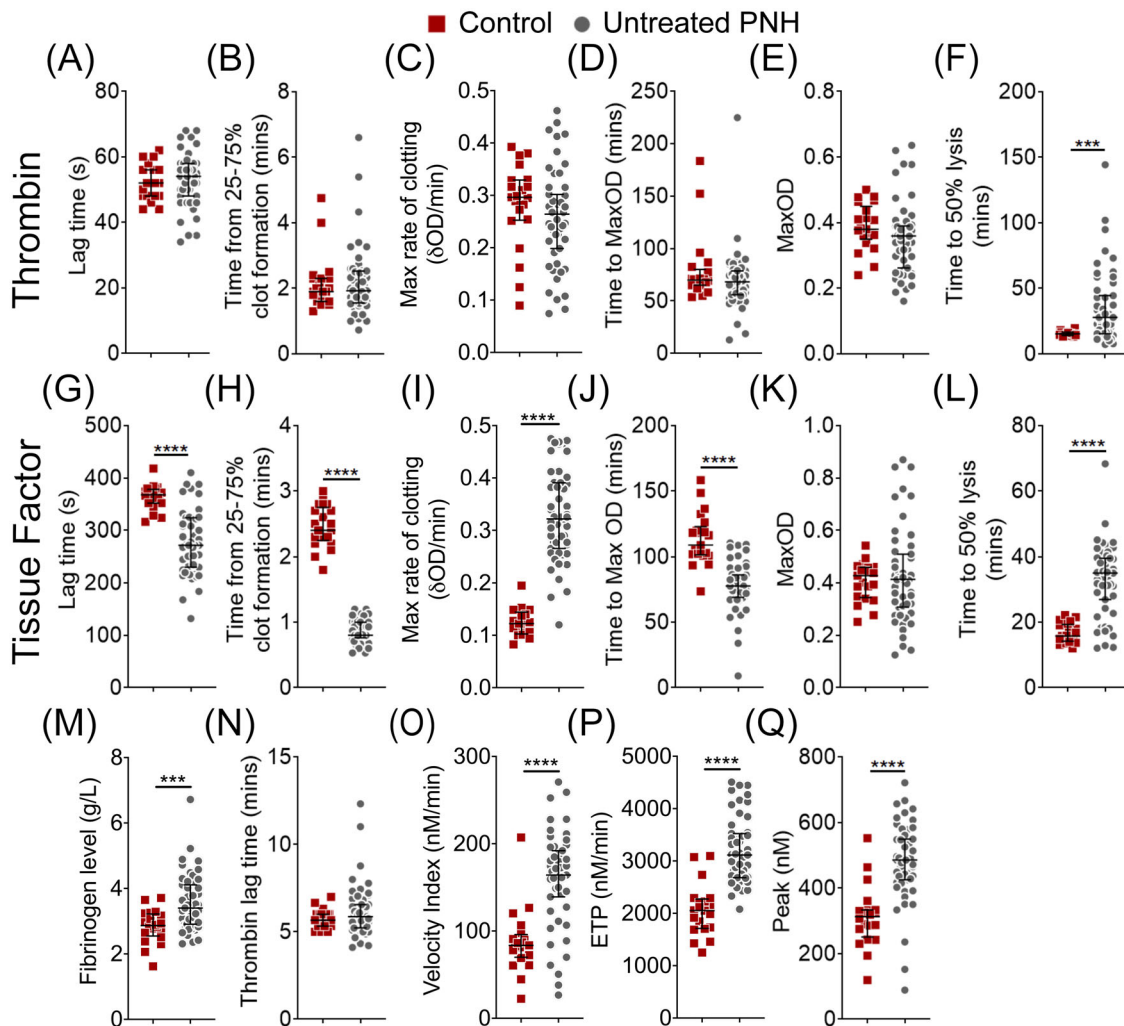
Clot formation and breakdown were analyzed by two separate turbidity and turbidity/lysis assays as described.<sup>17</sup> Investigated parameters were lag time, Maximum optical density (MaxOD), time to Max OD, time between 25% and 75% clotting, maximum clotting rate, time to 50% lysis (time from Max OD to 50% lysis), time between 25% and 75% lysis and maximum lysis rate. Triplicates were measured for each sample.

## 2.6 | Fibrinogen levels

Fibrinogen concentrations were measured by the Clauss method using a Start 4 hemostasis analyzer (Diagnostica Stago; Theale, UK) and the Fibri-prest automate kit (Diagnostica Stago; Theale, UK) according to the manufacturer's instructions.

## 2.7 | Thrombin generation

Thrombin generation was measured using the Calibrated Automated Thrombogram (CAT) method (Thrombinoscope BV, The Netherlands). Data was collected using the thrombinoscope software (Thrombinoscope BV, The Netherlands) and curves were automatically calculated by the



**FIGURE 2** The effect of PNH on clot formation and breakdown, fibrinogen levels and thrombin generation. The effect of PNH on different parameters of thrombin or tissue factor triggered clot formation and breakdown, fibrinogen levels and thrombin generation were assessed. A-F, thrombin, G-L, tissue factor. A and G, Lag time (s). B and H, Time from 25%-75% clotting (minutes). C and I, Max rate of clotting ( $\delta\text{OD}/\text{min}$ ). D and J, Time to MaxOD (mins). E and K, MaxOD. F and L, Time to 50% lysis (mins). M, Fibrinogen levels (g/L). N, Thrombin lag time (mins). O, Velocity index (rate of thrombin generation, nM/min). P, ETP (nM/min). Q, Peak thrombin generation (nM). Dunn's multiple comparisons test, error bars - median and IQR. ETP, Endogenous thrombin potential; OD, optical density; PNH, paroxysmal nocturnal hemoglobinuria. \*\*\* $P \leq .001$ , \*\*\*\* $P \leq .0001$  [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

analysis software, correcting for inner filter effect, substrate consumption and  $\alpha$ 2-macroglobulin-thrombin activity.

## 2.8 | Data analysis

Statistical tests and graphical representation of data were performed using GraphPad Prism v7 (La Jolla, CA, USA). Statistical significance was taken as  $P < .05$ . Distribution of data was tested using the D'Agostino and Pearson normality test. Continuous parametric data was presented as mean  $\pm$  SD, continuous non-parametric data as median and interquartile ranges and categorical data as number of subjects (% of total). Differences between control, untreated PNH and eculizumab treated groups were analyzed using Kruskal-Wallis test followed by a Dunn's multiple comparisons test for continuous data and Pearson's chi-square for categorical data. Correlations between parameters were analyzed using Spearman's rank correlation tests.

## 3 | RESULTS

### 3.1 | Patient demographics

Demographics and clinical characteristics of the PNH patients ( $n = 82$ ), and distribution of patients on ( $n = 51$ ) and off ( $n = 31$ ) treatment are presented in Table S1. As expected, patients on eculizumab had a larger median proportion of PNH cells of 97.6% than patients not on treatment with 34.4%, since patients with a higher proportion of PNH cells are more likely to be on treatment due to increased symptoms. Eculizumab prevents complement activation by inhibiting C5 cleavage and therefore intravascular hemolysis. Thus, patients on eculizumab presented with lower levels of LDH, 482 U/L, than patients not on eculizumab, 712 U/L (upper limit of normal 430 U/L), indicating decreases in intravascular hemolysis.<sup>18,19</sup> Median age of the healthy control group was lower than that of PNH patients on and off treatment (Table S2). However, none of the measured parameters in this study were found to correlate with age (Table S3).

### 3.2 | Clot structure in PNH patients

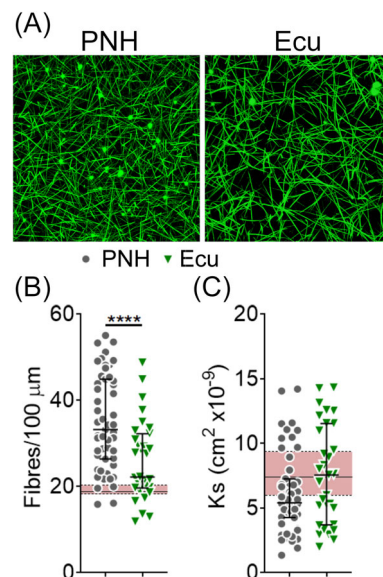
Due to the effects of eculizumab treatment on inflammation and complement activation, the effects of PNH on clot formation, structure and breakdown were analyzed in patients not on treatment. Changes in clot structure were investigated using confocal microscopy and permeation assays to assess clot density. Untreated patients with PNH presented with increased clot density when compared to healthy controls, 33.2 (IQR 26.4-44.9) vs 18.7 (18.3-20.2) fibers/100  $\mu$ m ( $P < .001$ ; Figure 1A, B). In agreement with this, PNH patients not on treatment also presented with lower clot porosity ( $K_s$ ) than healthy controls, 5.42 (4.3-7.3) vs 7.43 (6.0-9.3)  $\text{cm}^2 \times 10^{-9}$  ( $P = .005$ ; Figure 1C).

The effects of PNH on fibrin clot formation and breakdown were investigated using turbidity and lysis assays triggering clotting with

either thrombin or tissue factor (TF). When clotting was triggered with thrombin, no differences in lag time, time from 25%-75% of clot formation, maximum rate of clotting, time to MaxOD or MaxOD were seen between healthy controls and untreated PNH patients (Figure 2A-E; Table S4). However, when clotting was triggered with TF, untreated PNH patients presented with a shorter lag time, reduced time between 25% and 75% clot formation and increased maximum clot formation rate ( $P < .001$  for each) when compared to healthy controls, but with no difference in MaxOD (Figure 2G-K; Table S4).

Breakdown of fibrin clots was significantly delayed in untreated PNH patients when compared to healthy controls when clotting was triggered with thrombin or TF. In untreated PNH patients, time to 50% lysis and time from 25%-75% lysis were increased with both clotting triggers. Maximum lysis rate was decreased when clotting was triggered with TF when compared to healthy controls (Figure 2F, L; Table S4).

In view of the prothrombotic effects of PNH on fibrin clot structure and previously reported links between increased fibrinogen levels, thrombin generation and thrombosis risk,<sup>13</sup> fibrinogen levels and thrombin generation were investigated. Untreated PNH patients showed increased fibrinogen levels compared to healthy controls, 3.40 (IQR 2.91-4.11) vs 2.87 (IQR 2.55-3.22) g/L ( $P = .001$ ; Figure 2M). When thrombin generation was examined, no difference was found in the time taken to begin thrombin generation (Figure 2N; Table S5). However, significant increases in thrombin generation rate, ETP and



**FIGURE 3** The effects of eculizumab treatment on clot structure. A, Representative confocal microscopy images (Zeiss LSM880,  $\times 40$  oil immersion, NA 1.4,  $200 \times 200 \mu$ m) of plasma clots from patients on (Ecu) or off eculizumab treatment (PNH). B, Effects of eculizumab treatment on clot density (fibers/100  $\mu$ m). C, Effect of eculizumab treatment on clot porosity ( $K_s$ ,  $\text{cm}^2 \times 10^{-9}$ ). Shaded area represents healthy control data (solid line - median, dashed lines - IQR). Dunn's multiple comparisons test, Error bars - median and IQR. Ecu - eculizumab. \*\*\*\* $P \leq .0001$  [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



peak thrombin generation ( $P < .001$  for each) were observed in untreated PNH patients compared to healthy controls (Figure 2O-Q; Table S5).

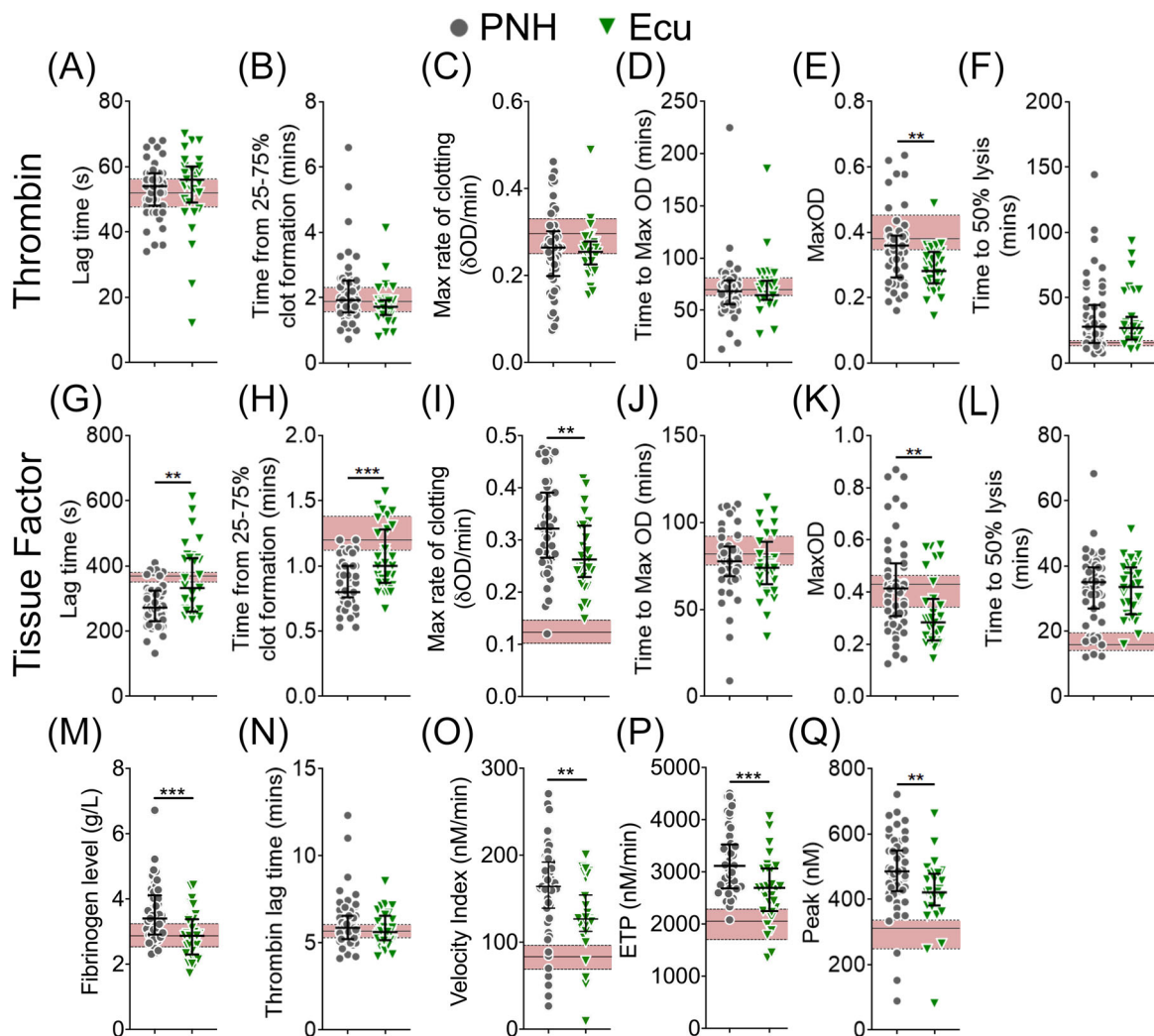
### 3.3 | Effects of disease severity

A number of studies have shown that PNH cell proportion determines thrombosis risk, with Moyo et al. calculating an odds ratio for thrombosis of 1.64 for every 10% increase in PNH cell proportion.<sup>8,20,21</sup> In our study there was positive correlation between increasing PNH granulocyte proportion and LDH levels ( $r = 0.86$   $P < .001$ , Figure S1) in untreated patients, indicating an increase in disease severity as proportion of PNH granulocytes increased. However, this

notwithstanding, no clear differences in clot structure, density or clot porosity (Figure S2A-C), clot formation and breakdown (Table S6), or fibrinogen levels and thrombin generation (Figure S2D-F) were seen as PNH granulocyte proportion increased in untreated patients.

### 3.4 | Eculizumab treatment

To assess if eculizumab treatment demonstrates anti-thrombotic effects on clot structure, clot formation and breakdown were investigated in plasma samples from PNH patients on and off treatment. The PNH patients on eculizumab treatment presented with less dense fibrin clots than patients not on treatment, 22.2 (IQR 19.7-32.2) vs 33.2 (IQR 26.4-44.9) fibers/100  $\mu\text{m}$  ( $P < .001$ ; Figure 3A-B). In



**FIGURE 4** The effect of eculizumab treatment on clot formation and breakdown, fibrinogen levels and thrombin generation. Differences in parameters of thrombin or tissue factor triggered clot formation and breakdown, fibrinogen levels and thrombin generation were assessed in patients on (Ecu) or off treatment (PNH). A-F, thrombin. G-L, tissue factor. A and G, Lag time (s). B and H, Time from 25%-75% clotting (minutes). C and I, Max rate of clotting ( $\delta\text{OD}/\text{min}$ ). D and J, Time to MaxOD (minutes). E and K, MaxOD. F and L, Time to 50% lysis (minutes). M, Fibrinogen levels (g/L). N, Thrombin lag time (minutes). O, Velocity index, rate of thrombin generation (nM/min). P, ETP (nM/min). Q, Peak thrombin generation (nM). Shaded area represents healthy control data (solid line - median, dashed lines - IQR). Dunn's multiple comparisons test, error bars - median and IQR. Ecu - eculizumab, OD - optical density, ETP - Endogenous thrombin potential. \*\* $P \leq .01$ , \*\*\* $P \leq .001$  [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

agreement with this, there was a trend towards a higher porosity of clot in patients on eculizumab compared to patients not on treatment, 7.56 (IQR 3.7-11.5) vs 5.42 (IQR 4.3-7.3)  $\text{cm}^2 \times 10^{-9}$ , but this did not reach significance due to the large spread of data ( $P = .099$ ; Figure 3C). These data suggest that treatment with eculizumab reduces fiber count and increases porosity helping to normalize clot structure.

When clotting was triggered with thrombin, no differences in lag time, time from 25%-75% of clot formation, maximum rate of clotting or time to MaxOD were seen between PNH patients on or off treatment (Figure 4A-D; Table S4). However, patients on eculizumab presented with a reduced MaxOD, 0.281 (IQR 0.243-0.340), compared to patients not on treatment, 0.359 (IQR 0.262-0.390,  $P = .009$ ), indicating that eculizumab treatment results in thinner fibrin fiber formation (Figure 4E; Table S4). When clotting was triggered with TF, treatment with eculizumab led to longer lag times, increased times between 25% and 75% clot formation, reduced maximum clot formation rates and reduced MaxOD, when compared to patients not on treatment (Figure 4G-K; Table S4). These data suggest that eculizumab contributes to reducing thrombotic risk by normalizing rates of clot formation and reducing fiber thickness.

Treatment with eculizumab appeared to have no effect on fibrinolysis, with patients on eculizumab showing no changes in time to 50% lysis, time from 25%-75% lysis and maximum lysis rate compared to patients not on treatment (Figure 4F,L; Table S4).

The PNH patients on treatment presented with decreases in fibrinogen levels compared to untreated PNH patients, 2.87 (IQR 2.30-3.38) vs 3.40 (IQR 2.91-4.11) g/L, returning them to similar levels as healthy controls ( $P = .001$ ; Figure 4M). No differences were found in the time taken to begin thrombin generation (Figure 4N). However, significant decreases in thrombin generation rate, ETP and peak thrombin generation were seen in eculizumab treated PNH patients compared to untreated PNH patients (Figure 4O-Q, Table S5). This is in agreement with a previous much smaller study on 11 patients with PNH, which investigated the effect of complement C5 inhibition with eculizumab over 90 days. It showed a reduction in thrombin-antithrombin complexes (TAT), a marker of thrombin generation.<sup>22</sup>

### 3.5 | Treatment time

Finally, we investigated the effects of eculizumab treatment time, which ranged from 2 weeks to 15 years. Treatment time was independent of PNH cell proportion, with no correlation between these parameters (Figure S3A), but LDH correlated negatively with treatment time (Figure S3B), suggesting intravascular hemolysis decreased with treatment time. Fibrinogen also decreased with time on treatment (Figure S4A). In addition, thrombin generation rate, ETP and peak thrombin all decreased with time on treatment (Figure S4B-D). In agreement with this, LDH levels were also found to correlate with fibrinogen, ETP and peak thrombin generation (Table S7). Fiber density and MaxOD were both negatively correlated with time on eculizumab treatment (Figure S4E-F). In addition to this, when clotting

was triggered with TF, clotting lag time, time from 25% to 75% clotting were found to increase, while MaxOD decreased, as the length of time on eculizumab increased (Figure S4G-I).

Taken altogether, our data show that patients with PNH present with a prothrombotic clot phenotype where clot formation occurs more rapidly, and fibrinolysis occurs more slowly. Furthermore, treatment with eculizumab leads to a time-dependent reduction in intravascular hemolysis (LDH), fibrinogen levels and thrombin generation, which in turn result in anti-thrombotic changes to clot structure.

## 4 | DISCUSSION

We explored changes in clot structure and the effects of anti-complement treatment with eculizumab in patients with PNH. Previous studies have demonstrated multiple factors contributing to a prothrombotic state in PNH, with a large proportion of these involving blood cells (neutrophils, monocytes, platelets and erythrocytes). However, we now show that humoral changes in clot structure may also contribute to thrombosis in PNH. Untreated PNH patients presented with higher fibrinogen and thrombin generation levels that subsequently lead to faster forming fibrin clots, which were harder to break down when compared to healthy controls. In untreated patients, increases in PNH granulocyte proportion led to increased LDH levels, indicating increased intravascular hemolysis. However, in platelet poor plasma no relationships were found between changes in PNH granulocyte proportion and thrombin generation, fibrinogen levels or fibrin clot formation, structure or breakdown. Patients treated with eculizumab had significantly decreased LDH, fibrinogen levels, thrombin generation and reduced clot formation rate and density. And, as time on eculizumab treatment increased, these anti-thrombotic effects augmented. These findings show that prior to treatment patients with PNH present with a prothrombotic fibrin clot phenotype compared to healthy controls. Increased fibrinogen levels and thrombin generation result in faster forming denser clots that are harder to break down. We therefore conclude that the anti-thrombotic effects of eculizumab treatment, in-part, are due to decreases in fibrinogen levels and thrombin generation with secondary changes in clot structure and function, that help to normalize the prothrombotic clot phenotype.

We are the first to investigate clot structure in PNH related thrombosis, in one of the largest collections of PNH patient plasma in the world. PNH is a pro-thrombotic disorder, with thrombosis being a major cause of mortality.<sup>11</sup> A number of studies have shown that increases in PNH cell proportion result in increased thrombosis risk,<sup>8,20,21</sup> and that uncontrolled complement activation is the most likely cause. Strong links between changes in clot structure and other thrombotic disease have previously been demonstrated,<sup>23,24,25</sup> with complement proteins also implicated.<sup>26,27</sup> It was thus hypothesized that increased thrombosis risk in patients with PNH may, in part, be due to the formation of pro-thrombotic clot structures and that treatment with eculizumab would lead to a normalization of clot structure decreasing thrombosis risk.

Results from platelet-poor plasma assays showed untreated patients with PNH had elevated fibrinogen concentration and thrombin generation and faster forming clots, that were denser and more difficult to breakdown. This is in line with many thrombotic diseases where strong links have been found between thrombosis risk, fibrinogen levels, thrombin generation and clot structural changes.<sup>13,28,29</sup> However, no links between the PNH cell proportion and clot structure, fibrinogen concentration or thrombin generation were found in untreated patients. This was unexpected as previous studies showed that increases in PNH granulocytes associate with increased thrombosis risk.<sup>8</sup> Furthermore, a number of cardiovascular studies have also shown correlations between changes in clot structure and disease severity.<sup>15,17</sup>

Our data suggest that untreated PNH patients have a prothrombotic fibrin clot phenotype regardless of their PNH granulocyte proportion. This also indicates that the previously reported increase in thrombosis risk with increasing PNH granulocyte proportion is most likely due to the cellular (rather than humoral) components of the blood, as these were not present in our plasma-based assays.

The relationships between PNH and increased fibrinogen levels, thrombin generation and subsequent changes in clot structure may partly be explained by the increase in intravascular hemolysis that occurs in PNH. Increased intravascular hemolysis results in systemic vascular inflammation leading to increased inflammatory responses including amplified interleukin-6 (IL-6) release.<sup>30,31</sup> Interleukin-6 is known to regulate fibrinogen synthesis, with fibrinogen genes highly responsive to changes in IL-6 levels,<sup>32</sup> and it is thus likely that increases in IL-6 levels would also lead to increases in fibrinogen production in PNH.

We also showed that thrombin generation rate, peak thrombin generation and total thrombin generation (ETP) are all increased in untreated PNH patients. This differed from two previous studies that found no difference, or a reduction in thrombin generation compared to healthy controls.<sup>12,33</sup> This difference may be due to the inclusion of patients on other forms of anti-coagulation in those studies, for example, warfarin which vastly decreases thrombin generation, which were excluded in our study. The use of anticoagulants in the treatment of PNH is still important<sup>34</sup> and further studies should be carried out to explore the effectiveness of anti-coagulants in the treatment of thrombosis in PNH. The levels of many coagulation proteins are increased in inflammatory conditions.<sup>35</sup> And, in agreement with this Grünewald et al. showed increases in coagulation factors V, VIII and X in patients with PNH.<sup>12</sup> Elevations of these coagulation factors would help to explain the significant increases in thrombin generation in untreated PNH patients in our study. Furthermore, intravascular hemolysis is known to result in a pro-coagulant state through the formation of erythrocyte fragments. This leads to increased thrombin generation,<sup>36</sup> and could further exacerbate the prothrombotic clot structure in untreated patients with PNH. Finally, the increase in thrombin generation could also be partly attributed to increases in fibrinogen levels. A number of studies have highlighted links between thrombin generation and fibrinogen levels,<sup>37,38,39</sup> indicating that increased fibrinogen levels lead to increased thrombin generation.

Fibrin clot structure is dependent on many factors, for example increased fibrinogen levels and thrombin generation both lead to more

densely packed clots.<sup>40,41</sup> The increase in fibrinogen levels and thrombin generation in untreated PNH patients could in part explain the changes in clot structure seen in this study. These changes resulted in faster forming fibrin clots that have a denser structure resulting in a more prothrombotic state. Untreated PNH patients were also found to have reduced fibrinolysis, with prolonged lysis times and reduced rates of lysis. This could be due to the changes in clot structure, with many previous studies linking denser fibrin clots to prolonged lysis times.<sup>42,43,44</sup> In addition, a previous study found that patients with PNH presented with significantly decreased plasminogen levels, and increased plasmin/antiplasmin and tPA/PAI-1 complexes.<sup>12</sup> Decreased plasminogen levels and increased inhibition of tPA and plasmin would lead to extended lysis times.

Treatment with eculizumab resulted in clear anti-thrombotic effects, with patients on eculizumab presenting with decreased fibrinogen levels, reduced thrombin generation and slower forming clots with reduced fiber density. Furthermore, as the length of time on eculizumab increased, the levels of LDH, fibrinogen concentration, thrombin generation, rates of clotting and clot density further decreased.

The effects of eculizumab treatment on fibrinogen levels, thrombin generation and clot structure are most likely explained by the reversal of PNH induced inflammation. Eculizumab treatment led to significant decreases in LDH indicating a decrease in intravascular hemolysis and other symptoms, as demonstrated previously.<sup>9</sup> Weitz et al. have also demonstrated that eculizumab treatment leads to decreased IL-6 levels and intravascular inflammation.<sup>22</sup> A reduction in IL-6 would lead to a reduction in fibrinogen and other coagulation factors subsequently reducing thrombin generation. A number of previous studies have reported reductions in markers of thrombin generation (thrombin-anti-thrombin complexes and fragment 1 + 2) in PNH patients treated with eculizumab.<sup>22,45,46,47</sup> We further showed that within the treatment group there was an independent correlation of both ETP and peak thrombin with LDH and fibrinogen levels, suggesting that changes in thrombin generation may be linked to one or both of these. Negative correlation of eculizumab treatment time with LDH levels, fibrinogen levels and thrombin generation in our study indicated that as the length of eculizumab treatment increased, the levels of LDH, fibrinogen and thrombin generation decreased bringing them closer to normal levels. These findings extend data previously reported, showing that eculizumab has very good long term efficacy far beyond the previously described 62-week study,<sup>48</sup> and potentially points to an improved or accumulative effect of the drug over longer time periods.

The reduction of fibrinogen levels and thrombin generation brought about by eculizumab treatment would lead to a subsequent reduction in clotting rates and the formation of less dense clots. Interestingly, treatment with eculizumab did not, however, lead to a reduction in lysis times despite the reduction in clot density. This suggests that the increases in lysis times seen in PNH patients are unlikely due to changes in clot density, and are more likely due to reductions in plasminogen levels and increases in plasmin and tPA-inhibitor complexes.

Our study has a few limitations. The rarity of PNH makes it difficult to obtain large study numbers, but the recruited 82 patients for this study is one of the largest PNH sample sizes in the literature. An even greater number of patients both on and off treatment with a larger

spread of PNH granulocyte proportions would help to increase power of future studies. The addition of a control group in this study helped to show that clot structure in untreated PNH patients is different to individuals without PNH. Yet many PNH patients have underlying bone marrow disorders and so comparison to healthy controls may not be optimal. It is possible that two control groups may be required in future studies, a control group of patients with an underlying bone marrow disorder and a second control group with healthy individuals. However, such a study with multiple groups is practically difficult to conduct and would also increase the number of comparisons leading to possible type I or II errors. Finally, further studies using whole blood assays in PNH patients would help uncover the contribution of complement activation and PNH affected blood cells in clot formation, but this is practically challenging as fresh blood experimentation would be required.

In summary, we have shown that untreated PNH patients presented with increased levels of fibrinogen and thrombin generation, which subsequently led to faster forming fibrin clots that had a denser structure. Untreated PNH patient clots also showed resistance to fibrinolysis. No changes in fibrin clot formation or structure were seen as the proportion of PNH cells increased in untreated patients, suggesting these changes are independent of disease severity. Eculizumab treatment led to significant reductions in LDH levels, fibrinogen levels, thrombin generation, clot density and rate of clot formation. Furthermore, there was significant negative correlation between the length of time patients are treated with eculizumab and LDH levels, fibrinogen levels, thrombin generation, clot density and rate of clot formation. These findings indicate that patients with untreated PNH have a prothrombotic clot phenotype brought about by increased levels of fibrinogen and thrombin generation. The anti-thrombotic effects of eculizumab may in part occur through a reduction in terminal complement activity and/or intravascular hemolysis, which in turn leads to a less thrombotic clot structure.

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#### AUTHOR CONTRIBUTIONS

F.L.M. performed research, analyzed data, and wrote the paper; B. P.-Y., P.B., S.Q., E.L., and S.R.B. performed research and analyzed data; P.H., M.G., T.M., and D.C. performed data collection and reviewed the paper; D.P. performed flow cytometry; C.M. processed samples; D.J. N, A.H., and R.A.S.A. designed the research and co-wrote the paper.

#### CONFLICT-OF-INTEREST

P.H., M.G., T.M., and A.H. have received honoraria from Alexion Pharmaceuticals, Inc.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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