Complement-mediated haemolysis and the role of blood transfusion in paroxysmal nocturnal haemoglobinuria

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Introduction

Paroxysmal nocturnal haemoglobinuria (PNH) is a rare form of acquired chronic haemolytic anaemia with unique characteristics. From a clinical point of view the classical triad is that of intravascular haemolysis, thrombosis and cytopenias[1,2]. From the point of view of pathogenesis, PNH can be regarded as a non-malignant clonal disorder, in which a mutation of the X-linked *PIG-A* gene in a haematopoietic stem cell affects the its mature progeny in the peripheral blood, including erythrocytes, leucocytes and platelets[3]: this progeny is, therefore, referred to as a *PNH clone*[4]. In PNH patients the PNH clone co-exists with qualitatively normal blood cells, although in many cases these are a minority[5]. PNH has no gender predilection and an estimated population frequency of between 1 and 10 per million.

Molecular basis of paroxysmal nocturnal haemoglobinuria

The first biochemical abnormality to be demonstrated in red cells from patients with PNH was a deficiency of the membrane protein acetylcholinesterase[6]. Subsequent studies showed that several more membrane proteins were missing or markedly decreased in the blood cells belonging to the PNH clone. From the serological point of view it is interesting that one of these (decay accelerating factor, see below) bears the Cromer blood groups[7]. In the 1980s it emerged that these deficient membrane proteins have one important feature in common: they are anchored to the cell surface through a glycosyl phosphatidyl inositol (GPI) molecule[8,9]. This suggested that the underlying biochemical abnormality would be in the GPI biosynthetic pathway, and it was indeed pinpointed at the step at which *N*-acetylglucosamine (NAcGlcN) is transferred onto phoshatidyl inositol[10]. Through expression cloning in a GPI-deficient cell line Taroh Kinoshita's group identified the *PIG-A* (phosphatidyl inositol glycan complementation group A) gene as being defective in PNH cells[11], and *PIG-A* somatic mutations were indeed identified in PNH patients[12]. *PIG-A* maps to the short arm of the X chromosome (band p22.1)[13]. The *PIG-A* product is a 484 amino acid protein which, with three other proteins, forms NAcGlcN transferase, the enzyme that catalyses the first step of the biosynthesis of the GPI anchor. Almost all the somatic mutations of the *PIG-A* gene identified so far in PNH patients are either frame-shift or nonsense mutations, only two are large deletions[14,15]. It should be noted that one of these inactivating mutations is sufficient to give rise on its own to the PNH phenotype because *PIG-A* is X-linked: as a result, only one allele is present in men, and only one allele is active in somatic cells in women. Very recently a PNH patient has been reported who had no *PIG-A* mutation: instead, she had a germ-line mutation of another gene (*PIG-T*) encoding a GPI biosynthetic enzyme, and a somatic mutation of the other *PIG-T* allele[16]. This patient provides a powerful confirmation of the notion that the critical biochemical failure in the PNH clone is in biosynthesis of the GPI anchor; in terms of frequency, this is likely to remain an exceptional situation[17].

Red cells in paroxysmal nocturnal haemoglobinuria and complement-mediated lysis

The complement (C) system is finely regulated by both soluble proteins in the plasma and membrane proteins on cells[18,19]. Red cells would be prime targets of complement activation, but they are protected by at least three major regulatory proteins: complement receptor-1 (CR1 or CD35), the decay accelerating factor (DAF or CD55), and the membrane inhibitor of reactive lysis (MIRL or CD59). The last two of these proteins, present on the surface of blood cells of all lineages, are GPI-linked: as such they are deficient on PNH red cells, which makes them exquisitely sensitive to lysis by activated complement. Specifically, CD55 controls the early complement pathway by inhibiting the C3 convertase and the C5 convertase[20]; CD59 interferes with the terminal effector pathway, by interfering with the incorporation of C9 onto the C5b-C8 complex, thus preventing formation of the membrane attack complex (MAC)[21].

Intravascular haemolysis in paroxysmal nocturnal haemoglobinuria

It is clear from the above why PNH patients have haemolytic anaemia. In this respect, although both

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of the GPI-linked complement-regulatory proteins deficient in PNH cells contribute to the complement susceptibility of PNH red cells, CD59 has generally been regarded as more important, for several reasons: (i) in PNH most of the haemolysis is intravascular (hence the haemoglobinuria): destruction of red cells in the bloodstream is mediated by MAC, and only CD59 can block the MAC; (ii) patients with a rare abnormality of the Cromer blood groups (the so-called Inab phenotype) are severely deficient in CD55, yet they do not have haemolytic anaemia[22-24]; (iii) in contrast, the few patients with genetically determined CD59 deficiency but normal CD55 do suffer from haemoglobinuria[25-27], and one of them also had thrombosis[25], reminiscent of PNH.

Multiple factors influence the severity of anaemia in paroxysmal nocturnal haemoglobinuria

As in every chronic haemolytic anaemia, in PNH the level of haemoglobin varies from patient to patient, and it also varies in time in each individual patient[28-30]. The reasons for this have not been fully elucidated, but we do recognise some of them.

1) Steady state *vs* paroxysms. Paroxysms of haemolysis, dramatically manifested through massive haemoglobinuria, have given the disease its name; however, it is important to be aware that intravascular haemolysis continues all the time: a good laboratory marker of this is the level of lactat dehydrogenase (LDH) in the serum. The simplest explanation for chronic intravascular haemolysis is that activation of complement takes place continuously at a low rate through the so-called "tick over" mechanism characteristic of the complement alternative pathway[31]. In contrast, when the classical pathway is activated through an antigen-antibody reaction C5 convertase is produced at a much higher rate: indeed, a paroxysm of haemoglobinuria (sometimes with a drop in haemoglobin of 4 g/dL or more in 24 hours) is often concomitant with an infection, even a minor one.
2) Patients with PNH usually have a markedly increased rate of erythropoiesis, with erythroid hyperplasia in their bone marrow and a high reticulocyte output. Thus they have an increased requirement for folic acid, of which they should receive daily supplements[32].
3) Since most of the haemolysis in PNH is intravascular, the large loss of haemoglobin through the urine entails continuous and considerable loss of iron[33]: thus, unlike with other chronic haemolytic anaemias the patient may become iron deficient. We have seen a gain in steady state haemoglobin level of 2-3 g/dL just through administration of iron (Notaro R and Luzzatto L, unpublished results).
4) As mentioned in the introductory paragraph, there is always an element of bone marrow failure lurking in a PNH patient, although this may be masked for years by the florid output of the PNH clone. However, sometimes there may be an evolution from florid PNH to aplastic anaemia in a few months: in this situation failure of red cell production may overlap with red cell destruction, or replace it altogether as a cause of anaemia (see also below, haemolysis *vs* bone marrow failure in PNH).
5) The introduction of eculizumab.

Ever since the Ham test[34] it was clear that haemolysis in PNH was complement-mediated, and it became predictable that inhibiting complement would be beneficial: but for over half a century there was no effective way to do this. In the late 1990s it was shown that anti-C5 antibodies were effective *in vitro*[35], and in 2007 a monoclonal anti-C5 antibody, eculizumab, was approved for the treatment of haemolytic PNH[36]. Eculizumab binds to C5 and prevents its cleavage by the C5 convertase: thus, the distal complement pathway is blocked and MAC formation cannot take place. For many patients with PNH eculizumab, which must be administered at regular 14 days intervals, has been a major advance in terms of control of intravascular haemolysis, relief from severe anaemia and improved quality of life[37-41].

The role of blood transfusion in paroxysmal nocturnal haemoglobinuria

The clinical trial that led to the licensing of eculizumab was conducted in patients who had haemolytic PNH severe enough to make them transfusion-dependent[38]. It is now clear that eculizumab is indicated even in a proportion of patients who are not necessarily transfusion-dependent in the steady state[32,42]. At any rate, it seems appropriate to consider separately the indications for blood transfusion in patients who are on eculizumab and in patients who are not on eculizumab.

a) *Patients who are not on eculizumab treatment*. As the name implies, PNH is characterised by exacerbations of a chronically ongoing haemolysis. Whenever there is an acute paroxysm, the clinical situation may be life-threatening (not unlike the situation with acute haemorrhage): in such cases there is an absolute indication for blood transfusion. In contrast, in the steady state, as in every chronic anaemia, we have to use educated judgement, particularly because we know that tolerance of anaemia is highly variable (see Table I) and in some cases blood transfusion may be hardly ever necessary (Figure 1A). The blood transfusion regime is, therefore, best tailored to the individual patient taking into account the rate of

Table I - Management of anaemia in PNH (other than with eculizumab).

1	In view of increased rate of erythropoiesis, give folic acid 5 mg/day.
2	Assess iron status by transferrin saturation index (TSI): if TSI <20%, give iron.
3	Determine "steady-state haemoglobin" (after correcting iron deficiency, if present)
4	Assess how steady-state level of anaemia is tolerated in terms of:
	- objective findings;
	- subjective feelings;
	- quality of life.
5	Based on (4), blood transfusion may be appropriate.
6	Blood transfusion requirement varies widely from patient to patient.
7	Blood transfusion requirement may also vary in the course of time in the same patient.
8	Washing red cells is no longer necessary: a good white cell filter is sufficient.
9	Irradiation of blood products is recommended if the patient is severely aplastic or has received immunosuppressive treatment.

fall in haemoglobin since the last count, the objective clinical assessment, and the subjective state of the patient, also in relationship to physical exertion. As a general guidance, a haemoglobin level of 7 g/dL is a yellow line, while 5 g/dL is a red line. We note that in PNH a blood transfusion will also dilute the percentage of PNH red cells in the patient's peripheral blood: this will reduce the proportion but not the absolute amount of red cells susceptible to acute haemolysis. Another important consideration is that, unlike with other transfusion-dependent chronic anaemias, iron overload is very infrequent, since so much iron is lost through the urine.

b) *Patients who are on eculizumab treatment.* Patients on eculizumab are in a newly achieved steady state, in which they can maintain haemoglobin levels higher than or equal to those that they had before starting eculizumab. As a result, the majority of patients who are on eculizumab no longer need blood transfusion (Figure 1B). However, approximately one quarter of patients who receive eculizumab regularly still require blood transfusion: the frequency is likely to be, for an individual patient, lower than it was before eculizumab was started[38,39,43,44] (Figure 1C). Paroxysms of haemolysis are rare in people taking eculizumab: they do occur sometimes either because an insufficient concentration of eculizumab is reached before the new infusion (pharmacokinetic breakthrough haemolysis)[45] or because massive activation of complement (via the classical pathway, as with an acute infection or with an inflammatory condition)[32,44,46] produces an excess of C3 convertase and C5 convertase activity that can displace eculizumab and thus cleave C5 (pharmacodynamic breakthrough haemolysis)[47]. Pharmacokinetic breakthrough haemolysis and the subsequent requirement for transfusion can usually be overcome by reducing the intervals between eculizumab doses or by increasing the dosage of eculizumab. Acute pharmacodynamic breakthrough haemolysis, although it produces a drop of haemoglobin, rarely requires blood transfusion, and it does not benefit from increasing eculizumab administration.

The most frequent reason for persistent requirement of blood transfusions in patients with PNH on eculizumab is chronic haemolysis with a persistently elevated reticulocyte count: the main mechanism is that a variable fraction of PNH red cells are opsonised by C3[43,48]. Indeed, when PNH red cells, having bound C3, are no longer lysed thanks to C5 blockade by eculizumab, they will yield a positive Coombs' test (which is regularly negative in PNH patients who are not on eculizumab), and these red cells will be subjected to what is in a way an iatrogenic extravascular haemolysis in the reticulo-endothelial system[43,49] (Figure 2). This phenomenon has a variable impact that fortunately becomes clinically relevant only in a minority of patients. Recently we have also learnt at least one reason why this phenomenon has considerable individual variability: this has to do with a genetic polymorphism of the (non-GPI-linked) complement receptor-1[50]. Those patients who are L/L homozygotes at this locus are three times more likely to remain transfusion-dependent on eculizumab compared to H/H homozygotes; H/L heterozygotes have an intermediate likelihood. In Japan a rare inherited mutation of C5 has been discovered which prevents the binding of eculizumab[51]: in patients with PNH who have this mutation transfusion requirement is unaffected and the drug should be discontinued.

Iron deficiency *vs* iron overload

As mentioned in the introduction, in PNH both the haemoglobinuria and haemosiderinuria favour the development of iron deficiency anaemia, which may require iron supplementation[32,42,52]. Clinically important iron loss from haemosiderinuria can occur even in the absence of gross haemoglobinuria. Therefore, iron overload, a well-known problem in patients receiving multiple blood transfusions, rarely occurs in PNH patients.

The situation is clearly different in those patients on eculizumab who still require blood transfusions: indeed, since intravascular haemolysis is abrogated, they no longer lose iron through the urine. As a consequence, they are susceptible to develop iron overload[46,53,54]: their iron status must be monitored and iron chelation instituted when appropriate.

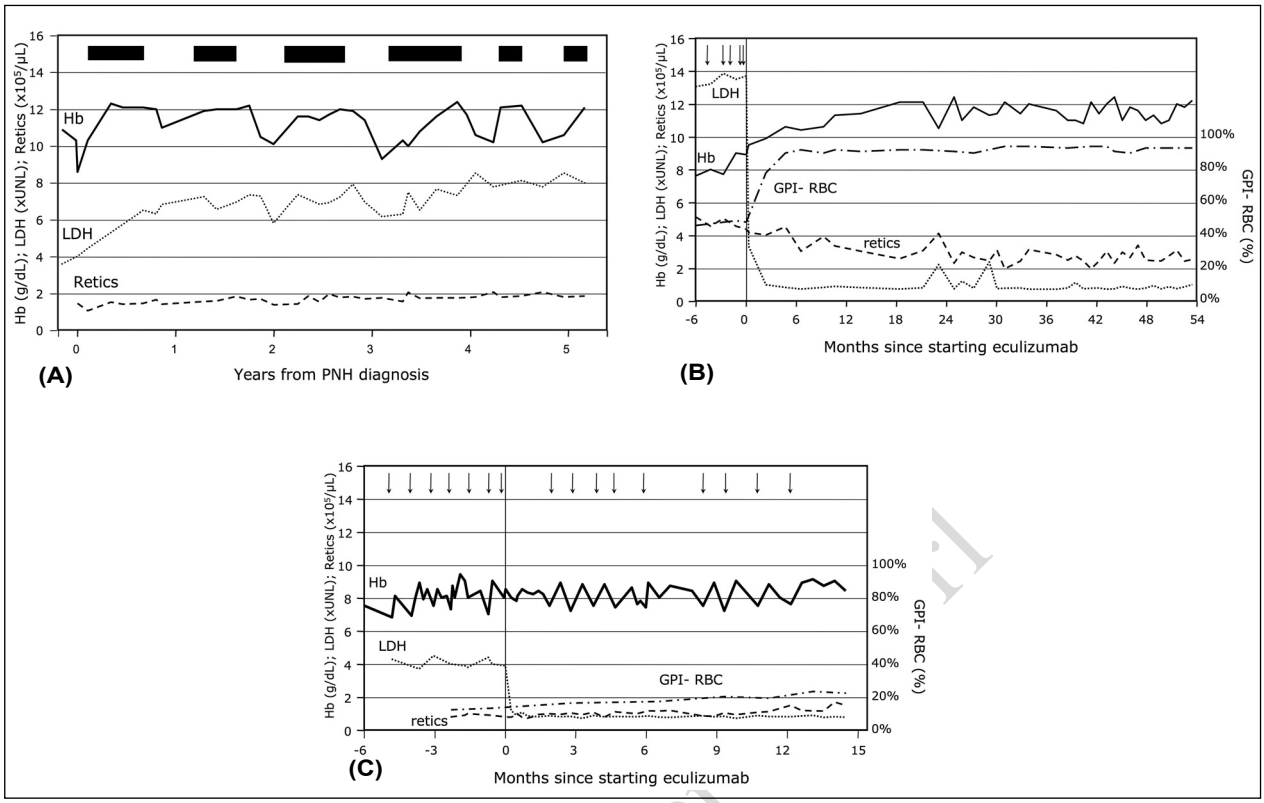

Figure 1 - Blood transfusion in the management of PNH: illustrative cases.

(A) This 35-year old male businessman with long-standing haemolytic PNH is subjectively well, is leading an active life, and has not yet needed a single blood transfusion. His intravascular haemolysis of considerable degree (see LDH levels) produces continued iron loss through the urine, necessitating periodic treatment with oral iron, without which his anaemia would be much more severe (whereby the patient would need blood transfusion eventually).

(B) This 48-year female office worker had a very high degree of intravascular haemolysis (see LDH levels) and was transfusion-dependent (average 2 units/month). On eculizumab her LDH dropped dramatically, the percentage of her GPI(−) red cells increased and, most importantly, she no longer needs blood transfusion.

(C) This 42-year old housewife has moderate to severe anaemia from a combination of haemolysis and probably inadequate bone marrow response (see reticulocytes). She was transfusion-dependent, requiring an average of 2-3 units/month. Unfortunately on eculizumab she remains transfusion-dependent, although she requires fewer units of red cells. The continued administration of eculizumab is justified by this, as well as by the full relief of subjective symptoms which, before she was on eculizumab, included frequent abdominal pain and dysphagia. Note that the increase in GPI(−) red cells appears spuriously poor, because these cells are diluted by the GPI(+) transfused red cells.

PNH: paroxysmal nocturnal haemoglobinuria; Hb: haemoglobin (g/dL); LDH: lactate dehydrogenase (×UNL: upper normal levels); Retics: reticulocytes (×10^5/μL). ↓ 2 units of packed red blood cells; GPI: glycosyl phosphatidyl inositol; GPI-RBC: GPI-negative red blood cells (%); ■: iron therapy.

Haemolysis *vs* bone marrow failure in paroxysmal nocturnal haemoglobinuria

A discussion of the bone marrow failure component of PNH falls outside the scope of this review. Suffice it to say that this component underlies cytopenias other than anaemia; and when the bone marrow failure component is prominent the patient is sometimes classified as having the PNH/aplastic anaemia (AA) syndrome[55]. It is becoming increasingly clear that PNH/AA is not really a separate entity, since AA can evolve into PNH, whereby at some intermediate point in time patients will have PNH/AA; conversely, a PNH patient may experience gradually increasing failure of bone marrow function, and at some point in time that patient may also be aptly described as having PNH/AA. Thus, in every PNH patient we must periodically try and assess both haemolysis and bone marrow function. With respect to haemolysis, the most helpful parameters are the LDH level if the patient is not on eculizumab and the bilirubin level if the patient is on eculizumab. With respect to bone marrow function, serial reticulocyte counts are the most useful criterion. For example, a reticulocyte count of 70,000/μL, although within the normal range, is inappropriately low if the haemoglobin level is 6.5 g/dL: indeed, it is highly suspicious of bone marrow failure. In practice, we must suspect bone marrow failure whenever a patient with PNH, whether or not

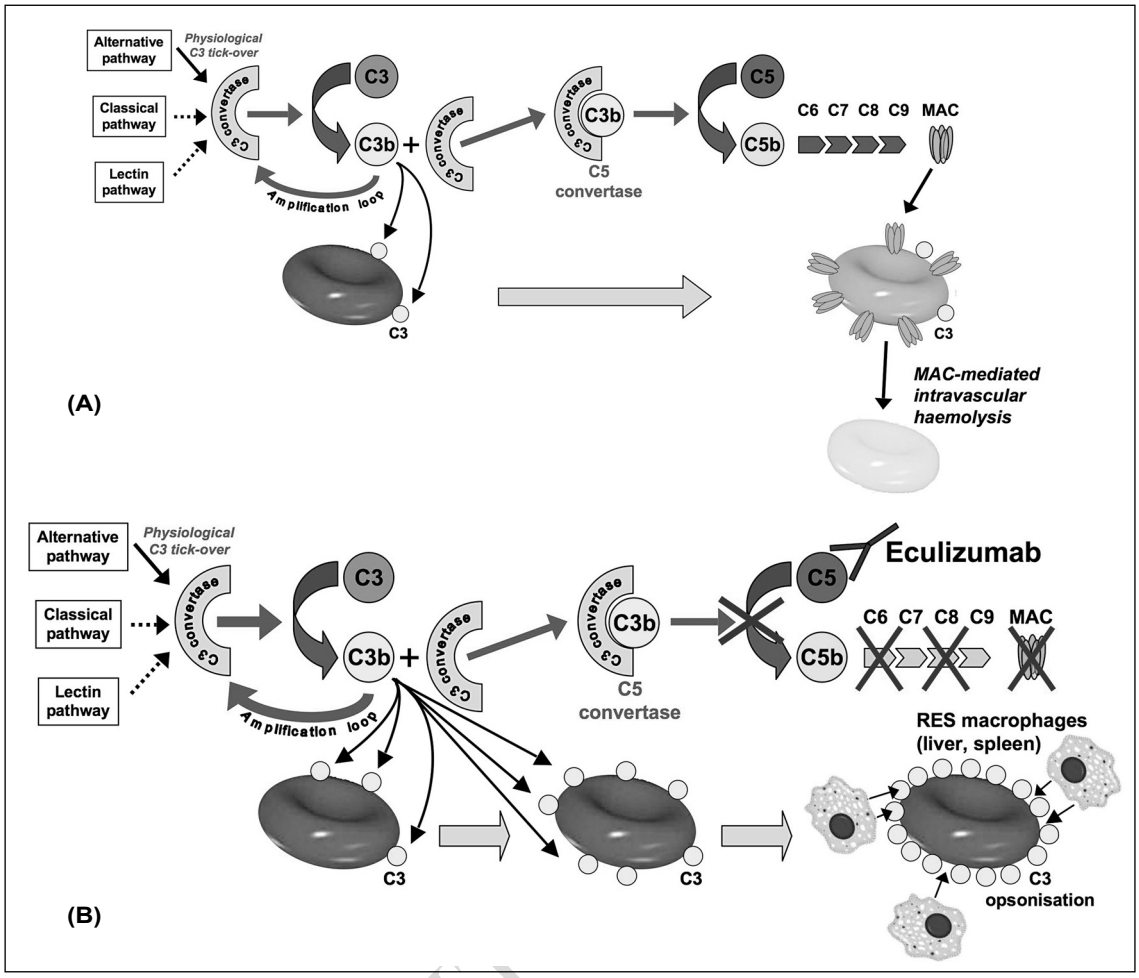

Figure 2 - The role of complement in intravascular and extravascular haemolysis in PNH.
(Modified from Luzzatto L, Risitano AM, Notaro R. Paroxysmal nocturnal hemoglobinuria and eculizumab. Haematologica 2010; 95: 523-6).
(A) Normal red cells are protected from complement activation and subsequent haemolysis by CD55 and CD59. PNH red cells, lacking CD55 and CD59, suffer from complement and will be lysed sooner or later by activated complement through formation of the MAC with consequent intravascular haemolysis.
(B) PNH red cells, in the presence of eculizumab, are protected from intravascular haemolysis because the inhibition of C5 prevents the formation of the MAC, but once opsonised by C3 they will become prey to macrophage with consequent extravascular haemolysis.
PNH: paroxysmal nocturnal haemoglobinuria; MAC: membrane attack complex; RES: reticuloendothelial system.

on eculizumab, who was not transfusion dependent, begins to require blood transfusions or the number of blood units required increases significantly. We must, therefore, constantly monitor the reticulocyte count, the granulocyte count and the platelet count.

A technical note

Since the purpose of blood transfusions in PNH patients is to correct anaemia, the blood component to be used is of course packed red cells. In the past a (non-haemolytic) transfusion reaction with fever and rigors was relatively common in these patients: in most cases this probably resulted from white cell antibodies produced in response to previous blood transfusions and it was therefore recommended that patients be transfused only with saline-washed red cells. Today, with the routine use of white cell filters, washing of red cells has become unnecessary and wasteful. On the other hand, any red cell incompatibility, even a minor one, must be avoided because an antigen-antibody reaction involving transfused (non-PNH) red cells would massively activate complement and thus cause haemolysis of the recipient PNH red cells as well. Blood units to be transfused ought, therefore, to be matched not only for ABO, but also for Cc, D, Ee, and Kell[56-58]. In the near future molecular red blood cell antigen matching extended to other blood groups will increase the safety and the effectiveness of transfusion therapy[59,60].

Conclusion

With the advent of eculizumab in the management of patients with PNH there has been a substantial decrease in the use of red cell transfusion; however, between 20 and 30% of patients will still require transfusion regularly, and any of them may need blood transfusion occasionally. We must also remember that in many countries eculizumab is not available at all, on account of its very high price, and in other countries it is only available for those patients who can afford that high price. Overall, red cell transfusion does, therefore, remain an essential item in the supportive therapy of PNH patients, both in the steady state and at the time of exacerbations of their chronic haemolytic anaemia.

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Keywords: paroxysmal nocturnal haemoglobinuria, complement activation, red cell transfusion, eculizumab, alternative complement pathway.

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