Achievements and Limitations of Complement Inhibition by Eculizumab in Paroxysmal Nocturnal Hemoglobinuria: The Role of Complement Component 3

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Abstract: Paroxysmal nocturnal hemoglobinuria (PNH) is a hematological disorder characterized by complement-mediated hemolytic anemia, thrombophilia and bone marrow failure. The clinical hallmark of PNH is evident chronic hemolysis due to the absence of the complement regulators CD55 and CD59 on PNH erythrocytes. Intravascular hemolysis drives the major clinical features of PNH, including anemia, hemoglobinuria, fatigue and other hemolysis-related disabling symptoms, such as painful abdominal crises, dysphagia and erectile dysfunction. A peculiar thromboembolic risk has been associated with the hemolysis in PNH, but its pathophysiologic cause remains unclear. The treatment of PNH has remained supportive until a few years ago, when the first complement inhibitor, designated eculizumab, became available. Chronic treatment with eculizumab results in sustained control of intravascular hemolysis, leading to hemoglobin stabilization and transfusion independence in half of the patients. However, residual anemia may persist in a substantial fraction of patients. Recent observations by different groups, including our own, have demonstrated that residual hemolysis may be due to persistent activation of the early phases of the complement cascade, leading to progressive C3-deposition on PNH erythrocytes and possible subsequent extravascular hemolysis through the reticuloendothelial system. Here we critically review the available clinical results of eculizumab treatment for PNH patients, pointing out the recent insights into the pathophysiology of the disease. We discuss the role of the different components of the complement cascade leading to hemolysis, in both the absence and presence of the terminal effector pathway inhibition by eculizumab. Finally, we provide a theoretical rationale for the development of novel strategies of complement inhibition which could in the future further improve on the already substantial efficacy of eculizumab.

Keywords: Paroxysmal nocturnal hemoglobinuria, intravascular hemolysis, eculizumab, complement component 3, extravascular hemolysis.

INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) [1-3] is a complex hematological disorder characterized by the clonal expansion of abnormal hematopoietic stem cell(s), which are unable to express glycosylphosphatidylinositol (GPI)-linked proteins on their surface due to a mutation in the X-linked *phosphatidylinositol glycan class A* (*PIG-A)* gene [4]. The hallmark of PNH is the chronic, complement-mediated, intravascular hemolysis, with subsequent hemoglobinuria and anemia; however, the typical clinical triad includes two other features: thromboembolic events (usually at venous sites) and bone marrow failure [1-3]. While the treatment options for PNH remained unsatisfactory for decades [5], with allogeneic stem cell transplantation being the only potentially curative option [6], the new century brought the availability of the complement inhibitor eculizumab [7], which has drastically changed the management of PNH patients. The short term benefits of eculizumab treatment afforded by controlling intravascular hemolysis was clear from the initial clinical trials [8-11]. In addition, it is anticipated that longer term treatment experience with eculizumab will show an impact on the natural history of the disease. In addition, the use of eculizumab has provided new insights into the pathophysiology of PNH [12]. Here we critically review the available clinical results of eculizumab treatment in PNH patients, especially focusing on biological observations which led to an improvement of our knowledge of PNH. In particular, we discuss the role of the early steps of the complement cascade, which in some PNH patients receiving eculizumab may lead to unexpected consequences, such as complement component 3 (C3) fragment-mediated extravascular hemolysis. We will also briefly introduce potential strategies designed to overcome such effects of complement inhibition by eculizumab, paving the way for future clinical investigations in patients with PNH.

THE PATHOPHYSIOLOGY OF PNH

PNH is due to an acquired mutation within *PIG-A* [4,13], an X-linked gene which encodes the N-acetyl glucosamine transferase essential to the first step of the GPI-anchor biosynthesis [14-16], subsequently impairing the expression of all GPI-linked proteins on the surface of affected cells [15,17]. The mutation is somatic, and occurs in one or a few hematopoietic stem cells; as a consequence, the aberrant

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phenotype is transmitted to all progeny blood cells – erythrocytes, platelets, granulocytes, monocytes and possibly lymphocytes. However, it is believed that the mutation itself is not sufficient to cause the disease, as demonstrated in mouse models [18,19]. This conclusion is also suggested by the observations that normal human subjects may harbor a small number of GPI-deficient cells without developing the disease [20]. Indeed, according to the commonly accepted dual step model of PNH pathophysiology [21], the expansion of the aberrant *PIG-A* mutated clone(s) requires the concomitant damage of the normal hematopoiesis; this damage would spare PNH stem cells, leading to a selective survival advantage for PNH hematopoiesis [22]. The actual nature of such damage is though to be immune-mediated [23] and due to a T cell-mediated attack on hematopoiesis similar to that causing immune-mediated aplastic anemia [24,25]. Thus, the autoimmune damage on hematopoiesis accounts for both the clonal expansion of PNH hematopoietic stem cells as well as for the bone marrow failure usually associated with PNH [22]. However, if on one side the expansion of PNH hematopoiesis may represent an escape preventing a more severe marrow failure, on the other side it leads to the PNH clinical phenotype as a consequence of the abnormal phenotype of progeny blood cells. This is especially true for the main clinical feature of PNH, intravascular hemolysis, and possibly also for thrombophilia, although for this latter disease manifestation the actual mechanism may be multifactorial and it has not been formally demonstrated.

Possible mechanisms leading to the thrombophilia of PNH include: (i) platelet activation due to uncontrolled surface complement activation; (ii) free hemoglobin released by intravascular hemolysis, which in turn depletes nitric oxide (NO) with subsequent dysregulation of platelet aggregation, endothelium activation and vessel wall tone [26]; (iii) thrombogenic properties of microvesicles released by red cells during hyperhemolysis and by the endothelium [27]; and (iv) fibrinolytic system impairment due to lack of membrane-bound uPAR and excess of soluble uPAR [28].

The Complement Cascade in the Pathophysiology of Hemolysis

The complement system is a key component of innate immunity, which has evolved to recognize both exogenous pathogenic microorganisms as well as injured self tissues, and to amplify adaptive immunity [29,30]. The complement system encompasses distinct functional pathways with unique mechanisms of activation, which then all merge into a common final effector mechanism, the cytolytic membrane attack complex (MAC). Thus, initiation of complement activation may occur along three different pathways – classical, alternative or lectin – which independently leads to activation of C3 and C5 convertases. While the classical and the lectin pathways require specific triggers to be activated, it has been known for decades that the complement alternative pathway (CAP) exhibits low-grade continuous activation due to spontaneous hydrolysis of C3 (the so-called *tick-over* phenomenon) [31,32]. In addition, some components of the CAP constitute an amplification mechanism (the so called *CAP amplification loop*), which amplifies complement activation regardless of the specific pathway that initially generates the first C3b molecule. Fine mechanisms have evolved to modulate the complement system, including membrane-bound proteins (complement receptor 1 [CR1], membrane cofactor protein [MCP], and the GPI-linked membrane proteins CD55 and CD59) as well as fluid-phase components, including factor I (FI) and factor H (FH) [33].

The lack of the complement regulators CD55 (also known as Decay Accelerating Factor, DAF) [34,35] and CD59 (or Membrane Inhibitor of Reactive Lysis, MIRL) [36,37] from the surface of PNH erythrocytes accounts for the most obvious manifestation of PNH, namely the intravascular hemolysis and hemoglobinuria. Indeed, it is well known that PNH erythrocytes are exquisitely vulnerable to complement activation, resulting in MAC assembly, which leads to chronic intravascular hemolysis. The hierarchical contribution of CD55 and CD59 to hemolysis suggests CD59 is the key molecule which, if absent, leads to lysis [38]; in contrast, redundant mechanisms (including CD59 itself) usually overcome the isolated deficiency of CD55 (as demonstrated in patients carrying the so-called Inab phenotype, who do not suffer from hemolysis) [39]. However, it should be reiterated that both complement activation and effector mechanisms are uncontrolled on PNH erythrocytes. Specifically, the lack of CD55 impairs regulation of the C3 convertases (regardless of the triggering pathway – classical or alternative) [40], leading to increased C3 activation and further progression along the subsequent steps of the cascade. It is also understood that low-grade spontaneous C3 tick-over leads to chronic CAP activation on the PNH erythrocyte surface [31,32]. Due to the lack of CD55, once activated by the CAP the complement cascade continues through to MAC assembly, finally coming to lysis due to the lack of CD59 inhibition of C8 and C9 incorporation into the MAC. Thus, given such mechanistic relationship between hemolysis and the complement cascade, the availability of complement inhibitors is a terrific breakthrough for the treatment of PNH patients.

THE CLINICAL FEATURES OF PNH

PNH is characterized by a triad of clinical features which is unique in medicine: intravascular hemolysis, thromboembolic events and cytopenia [1-3,5].

Complement-Mediated Intravascular Hemolysis

Hemolysis is found in all PNH patients, even if the degree may be variable due to the size of the PNH clone(s), as well as to the type of *PIG-A* mutation (missense mutations may lead to a partial deficiency of GPI-linked protein, the so-called type II PNH erythrocytes, which have a lower susceptibility to complement-mediated lysis in comparison to the type III PNH erythrocytes) [41]. Typically, the hemolysis is chronic, with possible exacerbations (the paroxysms) which occur due to massive complement activation, often in association with infections or other triggering events. Hemolysis leads to hemoglobinuria, urinary iron loss (seen as hemosiderinuria), and eventually anemia. The extent of anemia is very heterogeneous among patients, being also dependent from other factors such as compensatory erythropoiesis (which may be impaired in

patients with more severe marrow failure) or even iron/vitamin deficiency. As a result, anemia may be severe, requiring frequent transfusions, in some patients, or well-compensated in other cases. Besides anemia, it is now accepted that lysis may induce by itself specific disabling symptoms; they include malaise and fatigue (which may exceed that expected based on the low hemoglobin level), painful abdominal crises, dysphagia and erectile dysfunction [26]. All these latter symptoms, which usually occur in association with paroxysms of hemolysis, are though to be due to smooth muscle dystonia secondary to NO consumption [26].

Thrombophilia

Thromboses are an other key feature of PNH; they develop in about 40% of patients, often affecting specific sites (cerebral veins, hepatic veins [42], other splanchnic veins) which may cause devastating consequences [43-45]. In fact, thromboses are the leading cause of death for PNH patients [43-45]. Unfortunately, as the underlying pathogenic mechanisms are not fully understood [46,47], thromboses are unpredictable in PNH patients, and specific therapeutic interventions are mostly aimed at treating acute episodes [48] and then preventing frequent recurrences [5]. Primary prophylaxis by anti-coagulants [49] is not accepted by many physician due to a perceived unacceptable hemorrhagic risk for a majority of patients who may never develop a thrombotic event (and also to the incomplete prevention achieved by standard anti-coagulant therapies) [5,50].

Bone Marrow Failure

A third key clinical feature of PNH is cytopenia; as stated above, an underlying marrow disorder is embedded with the dual pathophysiology of the disease and with the PNH clone expansion. Thus, some degree of marrow failure is common in PNH patients, ranging from mild cytopenias to severe aplastic anemia [1,2]; of note, the specific picture of each individual patient may change during the disease course, with patients initially presenting with normal marrow function and subsequently developing more severe marrow failure, as well as patients initially diagnosed as aplastic anemia subsequently developing PNH [51]. According to a recent proposal of classification, distinct subtypes of PNH may be identified: classic PNH (which has hemolysis but not cytopenia), PNH with an underlying bone marrow disorder (with cytopenia and hemolysis) and subclinical PNH (presence of PNH cells without relevant hemolysis, usually in the context of other hematological disorders) [5]. However, distinct categories are hard to define for a disease with such an unpredictable presentation and evolution; furthermore, the clinical utility of classification depends on the ability to predict specific disease outcomes. In a recent large epidemiologic study from France it has been shown that the median survival of PNH patients is about 22 years from diagnosis, and that the main complications affecting the outcome were thrombosis, evolution to marrow failure and (rare) evolution to malignant disorders [44]. However, both survival and life-threatening complications did not differ among the PNH clinical subtypes [44].

THE TREATMENT OF PNH: THE ERA OF ECULIZUMAB

Eculizumab and Intravascular Hemolysis: Efficacy and Safety from the Registration Studies

The management of hemolysis of PNH, which had been palliative until 2000 by focusing on treating the anemic symptoms and episodic exacerbations rather than the causes underlying chronic hemolysis, has dramatically changed with the availability of eculizumab [50,52]. Eculizumab (Soliris®) is a humanized monoclonal antibody that targets the complement component C5 blocking its cleavage by C5 convertases, thereby preventing the production of the potent pro-inflammatory mediator C5a and the assembly of the MAC [6]. In the last few years eculizumab has been extensively investigated for the treatment of hemolysis in patients with transfusion-dependent PNH [8-11]. Safety and efficacy of eculizumab were initially established in one phase II pilot study [8] as well as in two phase III clinical studies (TRIUMPH and SHEPHERD) [9,10], and subsequently were confirmed in a common open-label Extension study [11]. Eculizumab was given intravenously dosed at 600 mg weekly for four weeks (loading phase), followed one week later by 900 mg fortnightly (maintenance phase); all patients were vaccinated against *Neisseria Meningiditis* at least two weeks before starting the treatment. After the initial pilot study, which provided proof-of-principle of effective blockade of intravascular hemolysis in eleven heavily transfused PNH patients [8], eculizumab was tested in a double-blind, placebo-controlled, multinational randomized trial which enrolled 86 patients [9]. The eligibility criteria included at least 4 red cell transfusions in the previous 12 months, a PNH type III erythrocyte population $\geq$10%, platelets $\geq$100 x 10^9/L, and lactate dehydrogenase (LDH) $\geq$1.5 times the upper limit of normal [9]. Treatment with eculizumab resulted in a dramatic reduction of intravascular hemolysis, as measured by LDH, leading to hemoglobin stabilization and transfusion independence in about half of the patients [9]. Control of intravascular hemolysis was found in all patients, and even cases not achieving transfusion independence showed a reduction of their transfusional needs. The effects of eculizumab on hemolysis were evident after the first administration, and lasted for the whole study period. In comparison to placebo, eculizumab also resulted in a significant improvement in fatigue and quality of life, as measured by validated questionnaires [9]. The safety profile of eculizumab was excellent, with negligible side effects and incidence of adverse events comparable to that of the placebo. These data were confirmed in the open-label phase III study SHEPHERD, which included a broader PNH population (minimum pretreatment transfusion requirement was one, and minimum platelet count requirement was 30 x 10^9/L) [10]. In the 96 patients enrolled in the study eculizumab resulted in a almost complete control of intravascular hemolysis, regardless of the pretreatment transfusion requirement, with transfusion independence achieved in half of the patients, and significant improvement in fatigue and quality of life [10]. The subsequent open-label Extension study enrolled a total of 187 patients who have previously completed one of the parent clinical trials [11].

The Extension study confirmed the efficacy and the safety of eculizumab with a longer follow up, confirming that the effects of eculizumab treatment on intravascular hemolysis were retained over time [11].

Eculizumab and Thrombophilia

In addition, as a secondary endpoint, the Extension study looked to the incidence of thromboembolic events in PNH patients on eculizumab, comparing the rate of thrombosis between the pretreatment and treatment periods in the same patients. The thromboembolism rate decreased from 7.37 to 1.07 events/100 patient-years after eculizumab treatment, with a 85% relative reduction [11]. This reduction was preserved even in patients on anticoagulants, suggesting that eculizumab may be the most effective agent to prevent thromboembolisms in PNH patients [11]. Whether eculizumab exerts its effect on thrombophilia of PNH directly, or through the blockade of intravascular hemolysis (e.g., by reduction of NO consumption or reduced release of procoagulant microvesicles), is still unknown. Recently, it has been reported that eculizumab treatment results in a significant decrease in plasma markers of coagulation pathway activation, reactive fibrinolysis and endothelial cell activation [53]. This finding suggests that the pathophysiology of thrombosis in PNH may involve multiple pathways, and the triggering events possibly affected by eculizumab have not been identified yet. However, if the protective effect of eculizumab on the thromboembolic risk will be confirmed in a long-term period, it is reasonable to anticipate that eculizumab may result in an improvement of survival of PNH patients. Such effect on survival has been recently shown in a limited cohort of patients [54].

Eculizumab and PNH: Any Additional Benefit?

More recently, it was reported that eculizumab may lead to additional beneficial effects for PNH patients. In particular, by counteracting NO consumption, eculizumab might reduce the risk of pulmonary hypertension (PH) [55]. This conclusion was mainly drawn through the 50% reduction of N-terminal pro-brain natriuretic peptide (NT-proBNP), which was found elevated at baseline in about 50% of PNH patients [55]. However, even if NT-proBNP can be considered a non-invasive marker for PH, possibly reflecting increased pulmonary vascular resistance and right ventricular dysfunction, it is usually utilized as prognostic marker in patients with proven diagnosis of PH [56]. Unfortunately this study does not include a direct estimation of pulmonary artery pressure by doppler echocardiography, making it uncertain as to whether these PNH patients exhibited clinically relevant PH. In an other study, eculizumab appeared to improve renal function of PNH patients, as measured by estimated glomerular filtration rate (eGFR), preventing possible chronic kidney disease (CKD) [57]. The authors report that before treatment a fraction of PNH patients may have decreased eGFR qualifying for stage 3-5 CKD (about 20%); eculizumab treatment resulted in an improvement of eGFR, and reduced the risk of major clinical kidney events [57]. Nevertheless, PH and CKD are not commonly described in PNH patients, and are not included among the common causes of death in the largest epidemiologic studies [40-42]. PH was observed in a single study only [58], while most reports dealing with renal function refer to acute renal failure secondary to paroxysms of hemolysis and hemaglobinuria [44,59,60]. Therefore, the real clinical impact of these finding has to be assessed in appropriate studies, and PNH should not be necessarily considered a progressive disease constantly leading to end-stage organ damage.

Eculizumab and Pregnancy

Pregnancy is a major problem for many young PNH patients. Due to both maternal and fetal risk of complications, usually secondary to thrombosis, most hematologists dissuade PNH women from pregnancy. Nevertheless, successful pregnancies have been described even without any anti-complement treatment [61]. Since eculizumab became available, three pregnancies have been reported in women receiving eculizumab throughout the gestation period; all of them led to healthy newborns, without any maternal complication [62,63]. Thus, even if eculizumab is formally not indicated in PNH pregnant women, and indeed the label for eculizumab classifies it as a pregnancy class C drug, common sense suggests that eculizumab should not be automatically withdrawn in the case of pregnancy, with careful consideration being given to the need to control the major causes of both maternal and fetal morbidity (intravascular hemolysis and subsequent anemia, and thrombophilia). It is still a matter of debate whether these data are sufficient to change our current counseling, letting highly motivated PNH women starting pregnancy during eculizumab treatment.

COMPLEMENT COMPONENT 3: EARLY COMPLEMENT ACTIVATION AND POSSIBLE C3-MEDIATED EXTRAVASCULAR HEMOLYSIS DURING ECULIZUMAB TREATMENT

C3-Mediated Extravascular Hemolysis During Eculizumab Treatment

Since the introduction of eculizumab in 2005, growing evidences suggested that its effect on MAC inhibition may unmask a biologically relevant and potentially pathogenic role for the early phases of the complement cascade [64]. We have recently documented that a novel clinically significant finding may appear in PNH patients receiving eculizumab, accounting for some portion of residual anemia and heterogeneous hematological benefit from the treatment [12]. In fact, while basically all patients achieve normal or almost normal LDH levels (pointing out an adequate control of intravascular hemolysis), only about a third reach a hemoglobin value above 11 gr/dL). In contrast, the remaining patients on eculizumab continue to exhibit moderate to severe (transfusion-dependent) anemia, in about equal proportions [12]. In our initial series of 56 PNH patients, we have demonstrated by flow cytometry that all the 41 PNH patients on eculizumab harbored C3 fragments bound to a substantial portion of their PNH erythrocytes (while all untreated patients did not) [12]. Our data were confirmed in an independent series by an other group, that exploited a direct antiglobulin test using C3d-specific anti-sera [65]. We concluded that membrane-bound C3 fragments work as opsonins on PNH erythrocytes, resulting in their

entrapment in the reticuloendothelial cells through specific C3 receptors and subsequent extravascular hemolysis [12,64,65]. This mechanism is supported by persistent reticulocytosis, hyperbilirubinemia and anemia in patients on eculizumab, and was also confirmed by *in vivo* erythrocyte survival study by ^{51}Cr labeling (which showed reduced survival and hepatosplenic ^{51}Cr uptake) [12].

The Complement Cascade Regulation During Eculizumab Treatment

Pathophysiologically, it is clear that such a mechanism becomes evident only when eculizumab prevents MAC-mediated hemolysis, allowing longer survival of PNH erythrocytes which continue to suffer from uncontrolled C3 convertase activation and C3 fragment deposition due to CD55 deficiency [64]. Indeed, the CAP is physiologically in a state of continuous activation because spontaneous (low-grade) hydrolysis of an internal thioester bond of C3 generates a C3b-like molecule, C3(H_2O); nascent C3(H_2O) is able to recruit factor B in forming (in the fluid phase) an unstable pro-C3 convertase. Once cleaved by factor D (generating C3(H_2O)Bb), this complex will in turn cleave additional C3 molecules to generate C3b, which binds predominantly to glycophorin A and activate (now in a membrane-bound phase) the CAP amplification loop [29-32]. On PNH erythrocytes, the lack of CD55 will allow this process (which is self-limiting on normal cells) to continue, leading to progressive CAP-mediated amplification, even in presence of eculizumab (which acts downstream). The reasons why only a fraction of PNH erythrocytes have membrane-bound C3, and why the proportion varies among patients, are not fully understood. Nevertheless, *in vitro* data support the concept that PNH erythrocytes are all susceptible to C3 deposition once exposed to conditions which cause complement activation [66]. We hypothesize that inter-individual differences in other physiological inhibitors (such as CR1 [67], complement FH [68] and complement FI) [69] may modulate the complement activation in a patient-specific fashion, leading to distinct patterns of C3 deposition. In addition, even more complex factors may drive the subsequent fate of C3-bound PNH erythrocytes; in fact, some patients may harbor substantially only C3-bound PNH erythrocytes, without showing a clinically relevant extravascular hemolysis [12,70]. At the moment, there is no ability yet to predict before starting eculizumab which patients will develop C3-mediated extravascular hemolysis.

Current Strategies to Overcome C3-Mediated Extravascular Hemolysis

C3 opsonization of PNH erythrocytes is a common phenomenon for PNH patients treated with eculizumab, even if the subsequent extravascular hemolysis may remain limited or well-compensated in most cases [64]. However, additional therapeutic strategies are needed for those patients developing a clinically relevant C3-mediated extravascular hemolysis, because they may continue to require frequent red cell transfusions, possibly developing subsequent iron overload (manuscript submitted). We reported a case managed by splenectomy [71], who achieved a substantial improvement of hemoglobin level without any medical complication; however, many physicians raise the concern that this approach may carry an increased life-long infectious risk [50]. In addition, the risk of intra- or peri-operatory complications (especially thrombosis, or hemorrhage in thrombocytopenic patients) might also argue against this therapeutic option. Very recently a group reported a single case where steroids were beneficial in controlling C3-mediated extravascular hemolysis [72]. We and others cannot confirm such observation in a larger series, and the well known side effects of long-term steroid use should advise against the use of steroids in PNH patients on eculizumab [70].

A Look into the Future of Complement Inhibition

The emergence of experimental and clinical evidence for CAP-initiated and C3 fragment-mediated extravascular hemolysis suggests that new treatment strategies appropriately targeting the early phases of the complement cascade should be assessed. The ideal agent should prevent the early phase of complement activation on PNH cells and defuse the amplification mechanisms (e.g., the CAP amplification loop). A systemic blockade of C3 activation through all pathways by a monoclonal antibodies (similar to the anti-C5 eculizumab) could be considered; however this approach may carry the risk of infectious and autoimmune complications secondary to a complete switching off of the complement system at this point. Recently, a different type of anti-C3 monoclonal antibody (the murine 3E7 and its chimeric-deimmunized derivative H17) has been developed, which recognizes activated C3 (C3b/iC3b), but not naïve, circulating C3; this antibodiy seems to affect the activity of the C3/C5 convertases of the CAP only, preserving the classical pathway [73]. These antibodies have been shown to be effective in inhibiting hemolysis of PNH erythrocytes *in vitro*. However, one have to consider that if C3/C5 convertases are localized on red cell surface, such an antibody may also work as an additional opsonine, leading to extravascular hemolysis *via* the Fc receptors. We are currently evaluating a different strategy, aiming to modulate the complement using a recombinant fusion molecule which combines two human proteins. Complement FH is a physiological complement inhibitor which modulates CAP-specific C3 and C5 convertase activity (but not classical pathway C3 and C5 convertases) predominantly in the fluid phase; it prevents C3 convertase formation, promoting its decay and working as a cofactor with complement FI to cleave C3b into the inactive fragment iC3b [68]. Thus, FH defuses both the spontaneous C3 tickover and the CAP amplification loop on normal and possibly on PNH erythrocytes [74]. In the aim of delivering FH activity locally at the site of complement activation, FH was fused with the iC3b/C3d-binding domain of complement receptor 2 (CR2). The resulting CR2-FH fusion protein has shown a dramatic inhibition of hemolysis of PNH erythrocytes *in vitro*, and further investigations are currently under way [75]. Once these or other next generation complement inhibitors proceed to clinical development, then we can determine whether such targeted inhibition should be additional or alternative to eculizumab. Indeed, the adequate control of C3, or both C3 and C5 activation on PNH red cells might make redundant the downstream blockade by eculizumab.

CONCLUSIONS

Since its initial description, PNH has remained for many investigators one of the most intriguing puzzles in medicine. Its major complement-dependent biochemical mechanisms were unraveled in the 1980s, and in the 1990s its genetic basis was defined. This last decade has seen the development of the first mechanism-based treatment for patients with PNH. The introduction of the complement inhibitor eculizumab has indeed resulted in a revolution for the management of PNH. In addition, eculizumab treatment has provided an opportunity to develop new insights into the pathophysiology of PNH. In fact, the blockade of the terminal effector MAC has brought to the forefront the potential pathogenic role of early complement activation of C3 itself. There are still many open questions about the role of C3 in PNH, such as the fine mechanisms regulating C3 deposition and further processing on PNH erythrocytes, as well as the reasons why, regardless of the level of clinically detectable C3 deposition, the clearance of C3 fragment-bound erythrocytes is heterogeneous among patients. However, the potential clinical relevance of the phenomenon suggests the need for strategies designed to deliver C3-targeted complement inhibition.

CONFLICT-OF-INTEREST DISCLOSURE

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REFERENCES

[1] Dunn, D.E.; Liu, J.M.; Young, N.S. 2000. Paroxysmal nocturnal hemoglobinuria. In: Young NS (ed). Bone Marrow Failure Syndromes. Philadelphia: W.B. Saunders Company. p 99-121.

[2] Rotoli, B.; Nafa, D.; Risitano, A.M. 2006. Paroxysmal nocturnal hemoglobinuria. In: Runge MD and Patterson C, ed. Principles of Molecolar Medicine, 2nd edition. Philadelphia, Humana Press. p 838-847.

[3] Parker, C.J. The pathophysiology of paroxysmal nocturnal hemoglobinuria. *Exp. Hematol*., **2007**, *35*, 523-33.

[4] Takeda, J.; Miyata, T.; Kawagoe. K.; Iida, Y.; Endo, Y.; Fujita, T.; Takahashi, M.; Kitani, T.; Kinoshita, T. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. *Cell*, **1993**, *73*, 703-11.

[5] Parker, C.; Omine, M.; Richards, S.; Nishimura, J.; Bessler, M.; Ware, R.; Hillmen, P.; Luzzatto, L.; Young, N.; Kinoshita, T.; Rosse, W.; Socié, G.; International PNH Interest Group. Diagnosis and management of paroxysmal nocturnal hemoglobinuria. *Blood*, **2005**, *106*, 3699-709.

[6] Santarone, S.; Bacigalupo, A.; Risitano, A.M.; Tagliaferri, E.; Di Bartolomeo, E.; Iori, A.P.; Rambaldi, A.; Angelucci, E.; Spagnoli, A.; Papineschi, F.; Tamiazzo, S.; Di Nicola, M.; Di Bartolomeo, P. Hematopoietic stem cell transplantation for paroxysmal nocturnal hemoglobinuria: long-term results of a retrospective study on behalf of the Gruppo Italiano Trapianto Midollo Osseo (GITMO). *Haematologica*, **2010**, *95*, 983-8.

[7] Rother, R.P.; Rollins, S.A.; Mojcik, C.F.; Brodsky, R.A.; Bell, L. Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria. *Nat. Biotechnol.*, **2007**, *25*, 1256-64.

[8] Hillmen, P.; Hall, C.; Marsh, J.C.; Elebute, M.; Bombara, M.P.; Petro, B.E.; Cullen, M.J.; Richards, S.J.; Rollins, S.A.; Mojcik, C.F.; Rother, R.P. Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria. *N. Engl. J. Med.*, **2004**, *350*, 552-559.

[9] Hillmen, P.; Young, N.S.; Schubert, J.; Brodsky, R.A.; Socié, G.; Muus, P.; Röth, A.; Szer, J.; Elebute, M.O.; Nakamura, R.; Browne, P.; Risitano, A.M.; Hill, A.; Schrezenmeier, H.; Fu, C.L.; Maciejewski, J.; Rollins, S.A.; Mojcik, C.F.; Rother, R.P.; Luzzatto, L. The complement inhibitor eculizumab in paroxysmal nocturnal hemoglobinuria. *N. Engl. J. Med.*, **2006**, *355*, 1233-43.

[10] Brodsky, R.A.; Young, N.S.; Antonioli, E.; Risitano, A.M.; Schrezenmeier, H.; Schubert, J.; Gaya, A.; Coyle, L.; de Castro, C.; Fu, C.L.; Maciejewski, J.P.; Bessler, M.; Kroon, H.A.; Rother, R.P.; Hillmen, P. Multicenter phase III study of the complement inhibitor eculizumab for the treatment of patients with paroxysmal nocturnal hemoglobinuria. *Blood*, **2008**, *114*, 1840-47.

[11] Hillmen, P.; Muus, P.; Dührsen, U.; Risitano, A.M.; Schubert, J.; Luzzatto, L.; Schrezenmeier, H.; Szer, J.; Brodsky, R.A.; Hill, A.; Socié, G.; Bessler, .M.; Rollins, S.A.; Bell, L.; Rother, R.P.; Young, N.S. Effect of the complement inhibitor eculizumab on thromboembolism in patients with paroxysmal nocturnal hemoglobinuria. *Blood*, **2007**, *110*, 4123-8.

[12] Risitano, A.M.; Notaro, R.; Marando, L.; Serio, B.; Ranaldi, D.; Seneca, E.; Ricci, P.; Alfinito, F.; Camera, A.; Gianfaldoni, G.; Amendola, A.; Boschetti, C.; Di Bona, E.; Fratellanza, G.; Barbano, F.; Rodeghiero, F.; Zanella, A.; Iori, A.P.; Selleri, C.; Luzzatto, L.; Rotoli, B. Complement fraction 3 binding on erythrocytes as additional mechanism of disease in paroxysmal nocturnal hemoglobinuria patients treated by eculizumab. *Blood*, **2009**, *113*, :4094-100.

[13] Miyata, T.; Takeda, J. ; Iida, Y. ; Yamada, N. ; Inoue, N. ; Takahashi, M. ; Maeda, K. ; Kitani, T. ; Kinoshita, T. The cloning of PIG-A, a component in the early step of GPI-anchor biosynthesis. *Science*, **1993**, *259*,1318-1320.

[14] Hirose, S.; Ravi, L.; Prince, G.M.; Rosenfeld, M.G.; Silber, R.; Andresen, S.W.; Hazra, S.V.; Medof, M.E. Synthesis of mannosylglucosaminylinositol phospholipids in normal but not paroxysmal nocturnal hemoglobinuria cells. *Proc. Natl. Acad. Sci. U S A*, **1992**, *89*, 6025-9.

[15] Takahashi, M.; Takeda, J.; Hirose, S.; Hyman, R.; Inoue, N.; Miyata, T.; Ueda, E.; Kitani, T.; Medof, M.E.; Kinoshita, T. Deficient biosynthesis of N-acetylglucosaminyl-phosphatidylinositol, the first intermediate of glycosyl phosphatidylinositol anchor biosynthesis, in cell lines established from patients with paroxysmal nocturnal hemoglobinuria. *J. Exp. Med*., **1993**, *177*, 517-21.

[16] Hillmen, P.; Bessler, M.; Mason, P.J.; Watkins, W.M.; Luzzatto, L. Specific defect in N-acetylglucosamine incorporation in the biosynthesis of the glycosylphosphatidylinositol anchor in cloned cell lines from patients with paroxysmal nocturnal hemoglobinuria. *Proc. Natl. Acad. Sci. U S A.*, **1993**, *90*, 5272-6.

[17] Boccuni, P.; Del Vecchio, L.; Di Noto, R.; Rotoli, B. Glycosyl phosphatidylinositol (GPI)-anchored molecules and the pathogenesis of paroxysmal nocturnal hemoglobinuria. *Crit. Rev. Oncol. Hematol.*, **2000**, *33*, 25-43.

[18] Rosti, V.; Tremml, G.; Soares, V.; Pandolfi, P.P.; 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*, 1028-1036.

[19] Keller, P.; Payne, J.L.; Tremml, G.; Greer, P.A.; Gaboli, M.; Pandolfi, P.P.; Bessler, M. FES-Cre targets phosphatidylinositol glycan class A (PIGA) inactivation to hematopoietic stem cells in the bone marrow. *J. Exp. Med*., **2001**, *194*, 581-589.

[20] Araten, D.J.; Nafa, K.; Pakdeesuwan, K.; Luzzatto, L. Clonal populations of hematopoietic cells with paroxysmal nocturnal hemoglobinuria genotype and phenotype are present in normal individuals. *Proc. Natl. Acad. Sci. U S A*, **1999**, *96*, 5209-5214.

[21] Rotoli, B.; and Luzzatto L. Paroxysmal nocturnal haemoglobinuria. *Baillieres Clin. Haematol.*, **1989**, *2*, 113-138.

[22] Luzzatto, L.; Bessler, M.; Rotoli, B. Somatic mutations in paroxysmal nocturnal hemoglobinuria: a blessing in disguise? *Cell*, **1997**, *88*, 1-4.

[23] Gargiulo, L.; Lastraioli, S.; Cerruti, G.; Serra, M.; Loiacono, F.; Zupo, S.; Luzzatto, L.; Notaro, R. Highly homologous T-cell receptor beta sequences support a common target for autoreactive T cells in most patients with paroxysmal nocturnal hemoglobinuria. *Blood*, **2007**, *109*, 5036-42.

[24] Karadimitris, A.; Manavalan, J.S.; Thaler, H.T.; Notaro, R.; Araten, D.J.; Nafa, K.; Roberts, I.A.; Weksler, M.E.; Luzzatto, L. Abnormal T-cell repertoire is consistent with immune process

underlying the pathogenesis of paroxysmal nocturnal hemoglobinuria. *Blood*, **2000**, *96*, 2613-20.

[25] Risitano, A.M.; Maciejewski, J.P.; Green, S.; Plasilova, M.; Zeng, W.; Young, N.S. In vivo dominant immune responses in aplastic anemia patients: molecular tracking of putatively pathogenic T cells by TCRβ-CDR3 sequencing. *Lancet*, **2004**, *364*, 353-363.

[26] Rother, R.P.; Bell, L.; Hillmen, P.; Gladwin, M.T. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. *JAMA*, **2005**, *293*, 1653–1662.

[27] Hugel, B.; Socié, G.; Vu, T.; Toti, F.; Gluckman, E.; Freyssinet, J.M.; Scrobohaci, M.L. Elevated levels of circulating procoagulant microparticles in patients with paroxysmal nocturnal hemoglobinuria and aplastic anemia. *Blood*, **1999**, *93*, 3451-6.

[28] Sloand, E.M.; Pfannes, L.; Scheinberg, P.; More, K.; Wu, C.O.; Horne, M.; Young, N.S. Increased soluble urokinase plasminogen activator receptor (suPAR) is associated with thrombosis and inhibition of plasmin generation in paroxysmal nocturnal hemoglobinuria (PNH) patients. *Exp. Hematol.*, **2008**, *36*, 1616-24.

[29] Müller-Eberhard, H.J. Molecular organization and function of the complement system. *Annu. Rev. Biochem.*, **1988**, *57*, 321-47.

[30] Holers, V.M. The spectrum of complement alternative pathway-mediated diseases. *Immunol. Rev.*, **2008**, *223*, 300-16.

[31] Pangburn, M.K.; Schreiber, R.D.; Müller-Eberhard, H.J. Formation of the initial C3 convertase of the alternative complement pathway. Acquisition of C3b-like activities by spontaneous hydrolysis of the putative thioester in native C3. *J. Exp. Med.*, **1981**, *154*, 856–67.

[32] Pangburn, M.K.; and Müller-Eberhard, H.J. Initiation of the alternative complement pathway due to spontaneous hydrolysis of the thioester of C3. *Ann. N. Y. Acad. Sci.*, **1983**, *421*, 291–8.

[33] Whaley, K.; and Ruddy, S. Modulation of the alternative complement pathway by β1H globulin. *J. Exp. Med.*, **1976**, *144*, 1147-63

[34] Nicholson-Weller, A.; March, J.P.; Rosenfeld, S.I.; Austen, K.F. Affected erythrocytes of patients with paroxysmal nocturnal hemoglobinuria are deficient in the complement regulatory protein, decay accelerating factor. *Proc. Natl. Acad. Sci. U S A.*,**1983**, *80*, 5066-70.

[35] Nicholson-Weller, A.; Spicer, D.B.; Austen, K.F. Deficiency of the complement regulatory protein, "decay-accelerating factor," on membranes of granulocytes, monocytes, and platelets in paroxysmal nocturnal hemoglobinuria. *N. Engl. J. Med.*, **1985**, *312*, 1091-7.

[36] Holguin, M.H.; Fredrick, L.R.; Bernshaw, N.J.; Wilcox, L.A.; Parker, C.J. Isolation and characterization of a membrane protein from normal human erythrocytes that inhibits reactive lysis of the erythrocytes of paroxysmal nocturnal hemoglobinuria. *J. Clin. Invest.*, **1989**, *84*, 7-17.

[37] Holguin, M.H.; Wilcox, L.A.; Bernshaw, N.J.; Rosse, W.F.; Parker, C.J. Relationship between the membrane inhibitor of reactive lysis and the erythrocyte phenotypes of paroxysmal nocturnal hemoglobinuria. *J. Clin. Invest.*, **1989**, *84*, 1387-94.

[38] Wilcox, L.A.; Ezzell, J.L.; Bernshaw, N.J.; Parker, C.J. Molecular basis of the enhanced susceptibility of the erythrocytes of paroxysmal nocturnal hemoglobinuria to hemolysis in acidified serum. *Blood*, **1991**, *78*, 820-9.

[39] Holguin, M..;, Martin, C.B.; Bernshaw, N.J.; Parker, C.J. Analysis of the effects of activation of the alternative pathway of complement on erythrocytes with an isolated deficiency of decay accelerating factor. *J. Immunol.*, **1992**, *148*, 498-502.

[40] Mold, C.; Walter, E.I.; Medof, M.E. The influence of membrane components on regulation of alternative pathway activation by decay-accelerating factor. *J. Immunol.*, **1990**, 145, 3836-41.

[41] Rosse, W.F.; and Dacie, J.V. Immune lysis of normal human and paroxysmal nocturnal hemoglobinuria (PNH) red blood cells. I. The sensitivity of PNH red cells to lysis by complement and specific antibody. *J. Clin. Invest.*, **1966**, *45*, 736-48.

[42] Hoekstra, J.; Leebeek, F.W.; Plessier, A.; Raffa, S.; Darwish Murad, S.; Heller, J.; Hadengue, A.; Chagneau, C.; Elias, E.; Primignani, M.; Garcia-Pagan, J.C.; Valla, D.C.; Janssen, H.L.; European Network for Vascular Disorders of the Liver. Paroxysmal nocturnal hemoglobinuria in Budd-Chiari Syndrome: findings from a cohort study. *J. Hepatol.*, **2009**, *51*, 696-706.

[43] Hillmen, P.; Lewis, S.M.; Bessler, M.; Luzzatto, L.; Dacie, J.V. Natural history of paroxysmal nocturnal hemoglobinuria. *N. Engl. J. Med.*, **1995**, *333*, 1253-1258.

[44] de Latour, R.P. ; Mary, J.Y. ; Salanoubat, C. ; Terriou, L. ; Etienne, G. ; Mohty, M. ; Roth, S. ; de Guibert, S. ; Maury, S. ; Cahn, J.Y. ; Socié, G. ; French Society of Hematology; French Association of Young Hematologists. Paroxysmal nocturnal hemoglobinuria: natural history of disease subcategories. *Blood*, **2008**, *112*, 3099-106.

[45] Nishimura, J.; Kanakura, Y.; Ware, R.E.; Shichishima, T.; Nakakuma, H.; Ninomiya, H.; Decastro, C.M.; Hall, S.; Kanamaru, A.; Sullivan, K.M.; Mizoguchi, H.; Omine, M.; Kinoshita, T.; Rosse, W.F. Clinical course and flow cytometric analysis of paroxysmal nocturnal hemoglobinuria in the United States and Japan. *Medicine (Baltimore)*, **2004**, *83*, 193-207.

[46] Markiewski, M.M.; Nilsson, B.; Ekdahl, K.N.; Mollnes, T.E.; Lambris, J.D. Complement and coagulation: strangers or partners in crime? *Trends Immunol.*, **2007**, *28*, 184-92.

[47] Ataga, K.I. Hypercoagulability and thrombotic complications in hemolytic anemias. *Haematologica*, **2009**, 94, 1481-4.

[48] McMullin, M.F.; Hillmen, P.; Jackson, J.; Ganly, P.; Luzzatto, L. Tissue plasminogen activator for hepatic vein thrombosis in paroxysmal nocturnal haemoglobinuria. *J. Intern. Med.*, **1994**, *235*, 85-9.

[49] Hall, C.; Richards, S.; Hillmen, P. Primary prophylaxis with warfarin prevents thrombosis in paroxysmal nocturnal hemoglobinuria (PNH). *Blood*, **2003**, *102*, 3587-91.

[50] Brodsky, R.A. How I treat paroxysmal nocturnal hemoglobinuria. *Blood*, **2009**, *113*, 6522-7.

[51] Scheinberg, P.; Marte, M.; Nunez, O.; Young, N.S. Paroxysmal nocturnal hemoglobinuria clones in severe aplastic anemia patients treated with horse anti-thymocyte globulin plus cyclosporine. *Haematologica*, **2010**, *95*, 1075-80.

[52] Parker, C. Eculizumab for paroxysmal nocturnal haemoglobinuria. *Lancet*, **2009**, *373*, 759-67.

[53] Helley, D.; de Latour, R.P.; Porcher, R.; Rodrigues, C.A.; Galy-Fauroux, I.; Matheron, J.; Duval, A.; Schved, J.F.; Fischer, A.M.; Socié, G.; French Society of Hematology. Evaluation of hemostasis and endothelial function in patients with paroxysmal nocturnal hemoglobinuria receiving eculizumab. *Haematologica*, **2010**, *95*, 574-81.

[54] Kelly, R.J.; Hill, A.; Arnold, L.M.; Brooksbank, G.L.; Richards, S.J.; Cullen, M.; Mitchell, L.D.; Cohen, D.R.; Gregory, W.M.; Hillmen, P. Long term treatment with eculizumab in paroxysmal nocturnal hemoglobinuria: sustained efficacy and improved survival. *Blood*, **2011**, Apr 1 [Epub ahead of print]

[55] Hill, A.; Rother, R.P.; Wang, X.; Morris, S.M.Jr.; Quinn-Senger, K.; Kelly, R.; Richards, S.J.; Bessler, M.; Bell, L.; Hillmen, P.; Gladwin, M.T. Effect of eculizumab on haemolysis-associated nitric oxide depletion, dyspnoea, and measures of pulmonary hypertension in patients with paroxysmal nocturnal haemoglobinuria. *Br. J. Haematol.*, **2010**, *149*, 414-25.

[56] Machado, R.F.; Anthi, A.; Steinberg, M.H.; Bonds, D.; Sachdev, V.; Kato, G.J.; Taveira-DaSilva, A.M.; Ballas, S.K.; Blackwelder, W.; Xu, X.; Hunter, L.; Barton, B.; Waclawiw, M.; Castro, O.; Gladwin, M.T; MSH Investigators. N-terminal pro-brain natriuretic peptide levels and risk of death in sickle cell disease. *JAMA*, **2006** , *296*, 310-8.

[57] Hillmen, P.; Elebute, M.; Kelly, R.; Urbano-Ispizua, A.; Hill, A.; Rother, R.P.; Khursigara, G.; Fu, C.L.; Omine, M.; Browne, P.; Rosse, W. Long-term effect of the complement inhibitor eculizumab on kidney function in patients with paroxysmal nocturnal hemoglobinuria. *Am. J. Hematol.*, **2010**, *85*, 553-9.

[58] Hill, A.; Wang, X.; Sapsford, R.J.; Rother, R.P.; Farrell, A.L.; Jessop, H.A.; McGawley, G.M.; Oxborough, D.L.; Pleasant, P.; Richards, S.J.; Arnold, L.M.; Buchanan, D.M.; Rollinson, S.; Gladwin, M.T.; Hillmen, P. Nitric oxide consumption and pulmonary hypertension in patients with paroxysmal nocturnal hemoglobinuria. *Blood*, **2006**, *106*, 305a.

[59] Kümpers, P.; Herrmann, A.; Lotz, J.; Mengel, M.; Schwarz, A. A blue kidney--chronic renal failure as a consequence of siderosis in paroxysmal nocturnal hemoglobinuria? *Clin. Nephrol.* **2006**, *66*, 210-3.

[60] Nair, R.K.; Khaira, A.; Sharma, A.; Mahajan, S.; Dinda, A.K. Spectrum of renal involvement in paroxysmal nocturnal

hemoglobinuria: report of three cases and a brief review of the literature. *Int. Urol. Nephrol.*, **2008**, *40*, 471-5.

[61] Fieni, S.; Bonfanti, L.; Gramellini, D.; Benassi, L.; Delsignore, R. Clinical management of paroxysmal nocturnal hemoglobinuria in pregnancy: a case report and updated review. *Obstet. Gynecol. Surv.*, **2006**, *61*, 593-601.

[62] Kelly, R.; Arnold, L.; Richards, S.; Hill, A.; Bomken, C.; Hanley, J.; Loughney, A.; Beauchamp, J.; Khursigara, G.; Rother, R.P.; Chalmers, E.; Fyfe, A.; Fitzsimons, E.; Nakamura, R.; Gaya, A.; Risitano, A.M.; Schubert, J.; Norfolk, D.; Simpson, N.; Hillmen, P. The management of pregnancy in paroxysmal nocturnal haemoglobinuria on long term eculizumab. *Br. J. Haematol.*, **2010**, *149*, 446-450.

[63] Marasca, R.; Coluccio, V.; Santachiara, R.; Leonardi, G.; Torelli, G.; Notaro, R.; Luzzatto, L. Pregnancy in PNH: another eculizumab baby. *Br. J. Haematol.*, **2010**, *150*, 707-708.

[64] Luzzatto, L.; Risitano, A.M.; Notaro, R. Paroxysmal nocturnal hemoglobinuria and eculizumab. *Haematologica*, **2010**, *95*, 523-6.

[65] Hill, A.; Rother, R.P.; Arnold, L.; Kelly, R.; Cullen, M.J.; Richards, S.J.; Hillmen, P. Eculizumab prevents intravascular hemolysis in patients with paroxysmal nocturnal hemoglobinuria and unmasks low-level extravascular hemolysis occurring through C3 opsonization. *Haematologica*, **2010**, *95*, 567-573.

[66] Sica, M.; Pascariello, C.; Rondelli, T.; Risitano, A.; Notaro, N. In vitro complement protein 5 (C5) blockade recapitulates the Complement protein 3 (C3) binding to GPI-negative erythrocytes observed in Paroxysmal Nocturnal Hemoglobinuria (PNH) patients on eculizumab. *Haematologica*, **2010**, *95*(s2), 196(a).

[67] Fearon, D.T. Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane. *Proc. Natl. Acad. Sci. U S A*, **1979**, *76*, 5867-71.

[68] Whaley, K.; and Ruddy, S. Modulation of the alternative complement pathway by β1H globulin. *J. Exp. Med.*, **1976**, *144*, 147-63

[69] Pangburn, M.K.; Schreiber, R.D.; Müller-Eberhard, H.J. Human complement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protein beta1H for cleavage of C3b and C4b in solution. *J. Exp. Med.*, **1977**, *146*, 257-70.

[70] Risitano, A.M.; Notaro, R.; Luzzatto, L.; Hill, A.; Kelly, R.; Hillmen, P. Paroxysmal Nocturnal Hemoglobinuria: Hemolysis Before and After Eculizumab. *N. Engl. J. Med.*, **2010**, *363*, 2270-72.

[71] Risitano, A.M.; Marando, L.; Seneca, E.; Rotoli, B. Hemoglobin normalization after splenectomy in a paroxysmal nocturnal hemoglobinuria patient treated by eculizumab. *Blood*, **2008**, *112*, 449-51.

[72] Berzuini, A.; Montanelli, F.; Prati, D. Hemolytic anemia after eculizumab in paroxysmal nocturnal hemoglobinuria. *N. Engl. J. Med.*, **2010**, *363*, 993-4.

[73] Lindorfer, M.A.; Pawluczkowycz, A.W.; Peek, E.M.; Hickman, K.; Taylor, R.P.; Parker, C.J. A novel approach to preventing the hemolysis of paroxysmal nocturnal hemoglobinuria: both complement-mediated cytolysis and C3 deposition are blocked by a monoclonal antibody specific for the alternative pathway of complement. *Blood*, **2010**, *115*, 2283-91.

[74] Ferreira, V.P.; and Pangburn, M.K. Factor H mediated cell surface protection from complement is critical for the survival of PNH erythrocytes. *Blood*, **2007**, 110, 1290-2.

[75] Risitano, A.M.; Holers, V.M.; Rotoli, B. TT30, a Novel Regulator of the Complement Alternative Pathway (CAP), Inhibits Hemolysis of Paroxysmal Nocturnal Hemoglobinuria (PNH) Erythrocytes and Prevents Upstream C3 Binding On Their Surface in An in Vitro Model. *Blood*, **2009**, *114*, 158a.

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