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Review Article

Multidisciplinary clinical management of paroxysmal nocturnal hemoglobinuria

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Abstract: Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, acquired disease caused by clonal expansion of one or more hematopoietic stem cell (HSC) lines due to a somatic mutation of the phosphatidylinositol glycan anchor (*PIG-A*) gene located on Xp22.1. PNH incidence is 1.5-2 cases per million of the population per year. PNH can affect multiple systems in the body and requires multidisciplinary clinical management. Patients can manifest with severe pancytopenia, life-threatening thrombosis affecting the hepatic, abdominal, cerebral, and subdermal veins, and high requirements for blood transfusion due to haemolytic anemia. PNH can also be associated with bone marrow failure. Advances in diagnostic techniques and a targeted therapeutic approach for PNH have emerged in the last two decades. Eculizumab, a promising humanized monoclonal antibody against C5, is the first approved therapy for PNH.

Keywords: Paroxysmal nocturnal hemoglobinuria, diagnosis, treatment, eculizumab

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare acquired clonal hematopoietic stem cell (HSC) disease characterized by intravascular chronic hemolytic anemia with acute episodes, bone marrow failure, and thrombosis. The clonal expansion of one or several HSCs with a somatic acquired mutation in the gene encoding *PIG-A* is the pathobiological mechanism that is underlying the development of PNH [1-3]. The true incidence of PNH is unknown but it is estimated at 1.5-2.0 cases per milion of the population per year, with a prevalence of 15.9 cases per milion of the population, similar to aplastic anemia (AA) [4]. Although PNH has a special place in the field of hematology, it is a multisystemic disease. The clinical course of PNH is usually chronic, with frequent flare-ups; spontaneous long-term remissions are rare. Median survival with conventional treatment (transfusions, steroids, immunosuppressive therapy etc.) is approximately 10 years, but is shorter if patients have thrombosis, renal deficiency, bone marrow failure or myelodysplasia [5-7]. The 5-year survival rate is 65% [8]. Eculizumab is a humanized monoclonal antibody directed against the terminal complement protein C5, and is the first successful targeted therapy for treatment of complement-mediated disease. The introduction of eculizumab has had a significant impact on the management of PNH. The drug has been shown to reduce hemolysis and improve symptoms and quality of life (QoL) of PNH patients [7-9].

The complement system and PNH

The complement system works through a number of serum proteins that may activate classi-

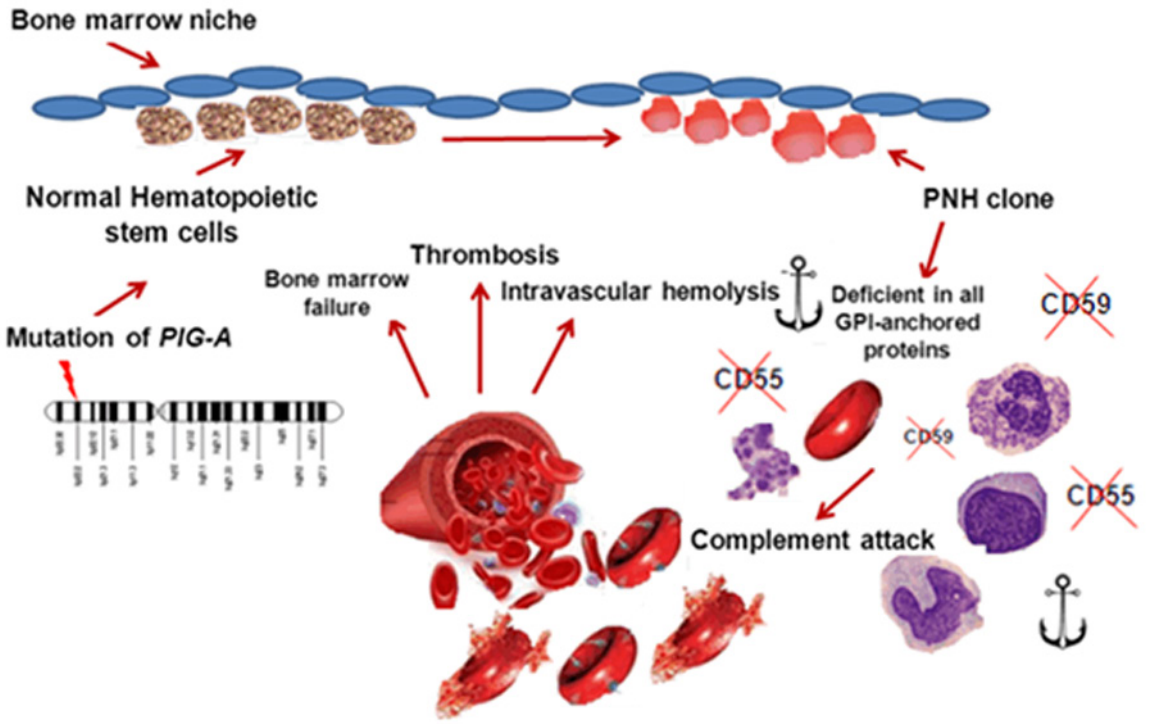

Figure 1. Pathogenesis of PNH. A somatic acquired mutation in the gene encoding PIG-A is the pathobiological mechanism underlying the development of PNH. Affected stem cells are deficient in all GPI-APs that serve as erythrocyte membrane-boundregulators of complement. Deficiency of CD55 and CD59 proteins accounts for the complement-mediated intravascular hemolysis and other clinical manifestations.

cal, alternative or lectin pathways that converge into one effector mechanism; the cytolytic membrane attack complex (MAC). The complement cascade undergoes disease-specific derangements that contribute to pathological outcomes. PNH, cold agglutinin disease (CAD) and hemolytic-uremic syndrome (HUS) are the clearest examples of complement-mediated diseases [10].

The *PIG-A* gene is required for synthesis of glycosyl phosphatidylinositol (GPI), which anchors certain proteins to the cell surface. These include CD55-decay accelerating factor (DAF), which inhibits the formation and the stability of the C3 convertase (both C3bBb and C4b2a), and CD59-membrane inhibitor of reactive lysis (MIRL), which interferes with the terminal effector, complement, blocking the incorporation of C9 onto the C5b-C8 complex. Affected stem cells (erythrocytes, granulocytes, monocytes, platelets and lymphocytes) are deficient in all GPI-anchored proteins (GPI-APs) that serve as erythrocyte membrane-bound regulators of complement. Deficiency of CD55 and CD59 proteins accounts for the complement-mediated intravascular hemolysis that is the hallmark of PNH (**Figure 1**) [11]. The hierarchical subscription of CD55 and CD59 to hemolysis indicates that CD59 is the key molecule in the prevention of lysis [12].

Clinical presentations

Patients with PNH can present with multisystemic clinical manifestations due to intravascular hemolysis, thrombosis and bone marrow failure (**Figure 2**) [13]. Symptoms are therefore often non-specific, ranging from loss of vision (due to retinal thrombosis), headache and nausea/vomiting (due to cerebral thrombosis), pulmonary hypertension (due to pulmonary embolism), anaemia, through to pain and swelling in the lower extremities (due to deep vein thrombosis), renal failure and erectile dysfunction.

Diagnosis of PNH

PNH is not a simple diagnosis. Detection of GPI-linked antigens on hematopoietic cells using monoclonal antibodies and flow cytometry analysis of peripheral blood cells are required

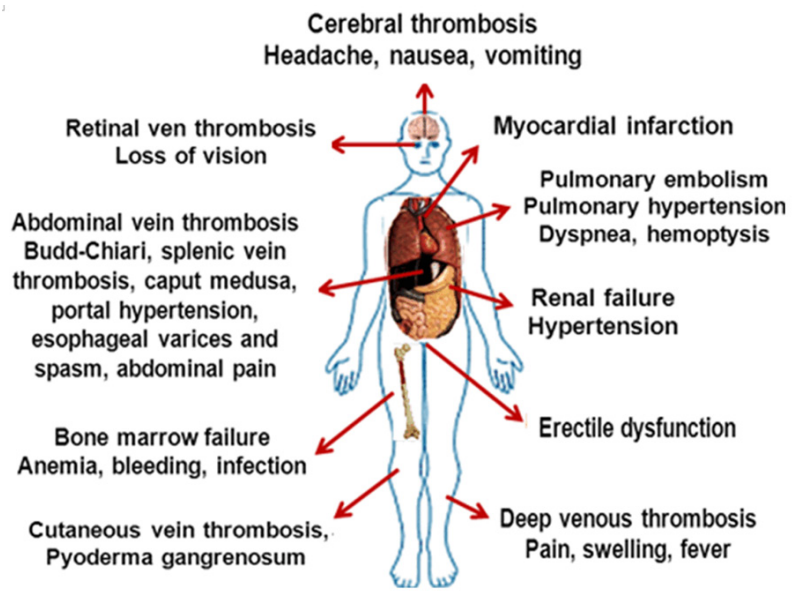

Figure 2. Clinical manifestations of PNH.

for the basis of a specific PNH diagnosis and classification [14]. Flow cytometry is also a tool for measuring the size of the PNH clone. Analysis of both erythrocytes and peripheral blood mononuclear cells is warranted because clone size will be underestimated if only erythrocytes are examined due to destruction of GPI-AP deficient cells by complement. Recent transfusions also diminish the ratio of erythrocyte clone size and can lead to inaccurate clone size measurements. In addition, specific PNH phenotypes are best established by detailed analyses of the erythrocyte population, initial complete blood count, biomarkers of hemolysis (lactate dehydrogenase [LDH], bilirubin, haptoglobin), and iron stores.

Flow cytometry

Flow cytometric analysis of CD59 and/or CD55 using antibodies directed against GPI-AP on peripheral blood cells are informative assays and most commonly used in the diagnosis of PNH [14]. But CD55 generally too dim, doesn't show good signal/noise ratio, only analysis of CD59 antibodies on anti-glycophorin A (CD235a) gated erythrocytes is used to identify erythrocyte PNH cone size. The advantage of flow cytometric PNH diagnosis on peripheral red blood cells is more accurate determination of the degree of GPI anchor deficiency (PNH Type III [complete deficiency], Type II [partial deficiency], and Type I [normal expression] clones). However, this method has low sensitivity due to the short half-life (20-45 days) of circulating PNH red blood cells, and it is not sensitive enough to detect small PNH clones (< 1%) in AA and MDS [13]. In contrast, granulocyte PNH clone gives more accurate estimate of PNH clone size and flow cytometric granulocyte analysis is accepted as gold standard for PNH diagnosis, Monocyte analysis is generally performed to confirm the granulocyte results. In white blood cell analysis assessments of the deficiency of GPI-linked proteins such as CD24, CD16 and CD66b on $CD15^+$ granulocytes and the deficiency of CD14 on $CD33^+$ monocytes is needed. However, bacterial lysin FLAER is the most versatile reagent for detecting PNH in all white blood cells, and demonstration of at least one GPI-linked protein deficiency in addition to FLAER is both sensitive and specific for the diagnosis of PNH [15]. A flare result of a PNH patient has shown in **Figure 3**.

Flow cytometric analysis of bone marrow cells is not diagnostic due to the presence of progenitor cells in the tissue samples. In addition, no significant correlation has been identified between cell marker expression and polymorphonuclear (PMN) leukocyte count, reticulocytosis, bilirubin and serum LDH [16].

Bone marrow examination

Bone marrow examination is not necessary for flow cytometry. It should only be performed to assess bone marrow failure and cytogenetics.

PNH and anemia

The absence of CD59 from the surface of PNH erythrocytes leads to chronic and acute flare-ups of intravascular haemolysis due to the aggregation of C9 into the C5b-8 complex, which produces a continuous state of complement MAC activation [17].

The deficiency of CD55 leads to extra-vascular haemolysis due to a partial reduction or complete loss of its function in the acceleration of the rate of destruction of membrane-bound

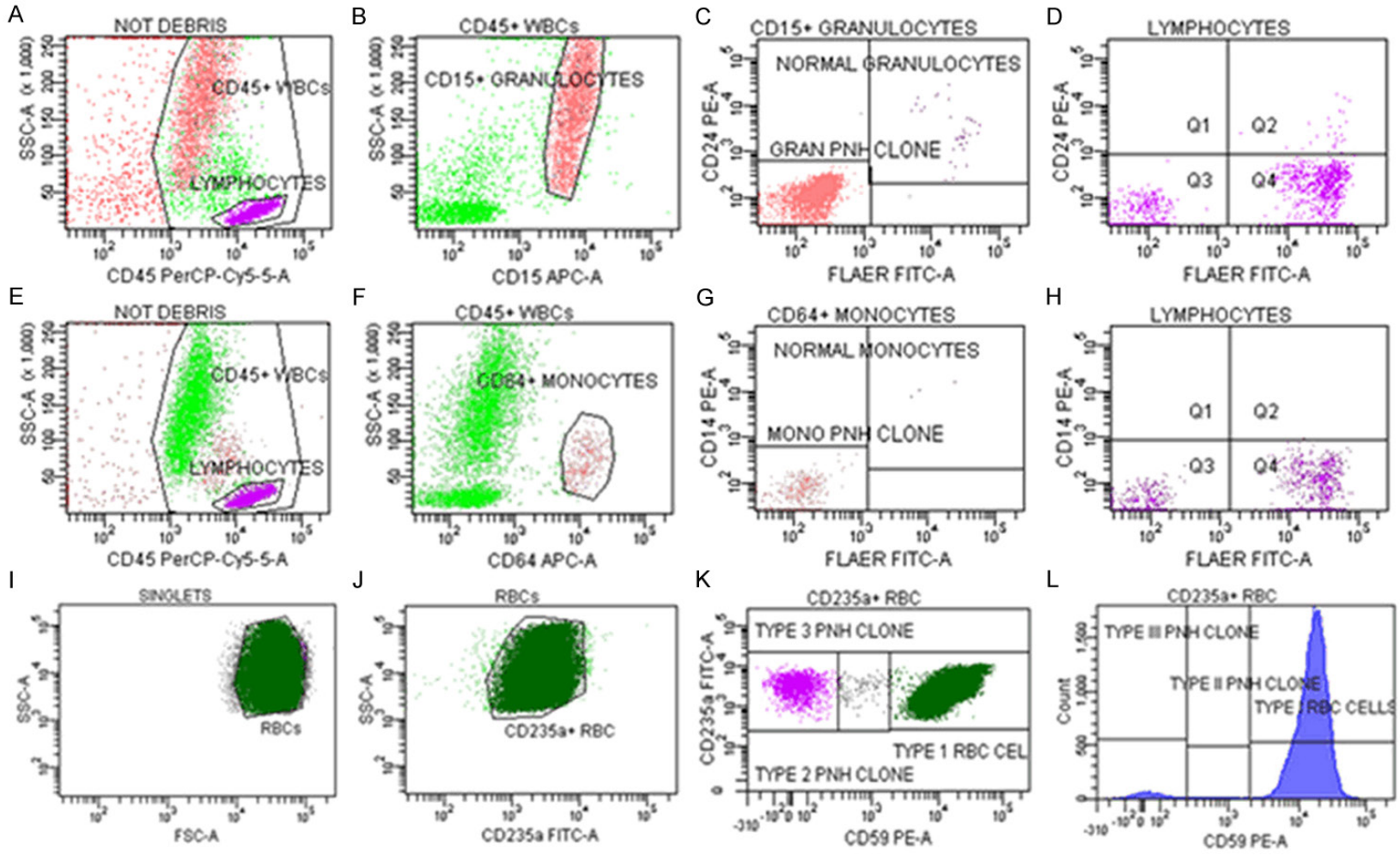

Figure 3. FLAER results of a patient with PNH. PNH testing on granulocytes using HSFC PNH testing; using SSC-CD45 graph, WBCs and Lymphocytes are gated (A). and $CD45^+$ WBCs are selected for the next graph: SSC-CD15 in which $CD15^+$ granulocytes are gated (B). Those granulocytes are analyzed based on their CD24, a GPI-linked molecule found on granulocytes, and FLAER expression (C). As you can see from (C), 98.7% of $CD15^+$ Granulocytes are double negative in terms of CD24 and FLAER which shows their lack of GPI expression and called as "PNH Clone". Lymphocytes which are gated on SSC-CD45 graph, can be evaluated also based on their CD24/FLAER pattern. Due to long spans of lymphocytes, we can see the smaller clone size (26.33% of Lymphocytes are lack of GPI) (D). PNH testing on monocytes using HSFC PNH testing; Using SSC-CD45 graph, WBCs and lymphocytes are gated (E) and $CD45^+$ WBCs are selected for the next graph: SSC-CD64 in which $CD64^+$ monocytes are gated (F). Those monocytes are analyzed based on their CD14, a GPI-linked molecule found on monocytes, and FLAER expression (G). 99.19% of $CD64^+$ monocytes are double negative in terms of CD14 and FLAER which shows their lack of GPI expression and called as "PNH Clone". Lymphocytes which are gated on SSC-CD45 graph, can be evaluated also based on their CD14/FLAER pattern. Due to long spans of lymphocytes, we can see the smaller clone

size (23.18% of lymphocytes are lack of GPI) (H). PNH testing on RBCs using HSFC PNH testing; using SSC-FSC log graph, RBCs are gated (I) and those RBCs are further analyzed based on their CD235a expression (J), a molecule which is found only on RBCs. Finally $CD235a^+$ RBCs are analyzed in terms of their CD59 expression. There are 3 types of cells present in PNH positive samples. Type 3 cells are completely negative for GPI and thus negative for CD59 expression. Type II cells partially express GPI and thus have dim-CD59 expression. Finally type I cells are healthy RBCs carrying GPI molecule and have bright CD59 expression (K and L).

C3-convertase. Extra-vascular haemolysis is lower because CD59 deficiency leads to more rapid red cell destruction [1]. Bone marrow failure is also a factor for anemia in PNH.

Renal deficiency in PNH

Renal involvement is usually not clinically apparent in PNH but damage may occur at any point in time. Renal failure is the reported cause of mortality in 8-18% of PNH patients [18]. Acute kidney injury (AKI) can arise in PNH due to the release of heme pigments, with subsequent hemoglobinuria, due to intravascular haemolysis. In addition, hemosiderosis-iron deposition in the kidneys can cause proximal tubular dysfunction due to chronic hemolysis [19]. Chronic renal dysfunction can arise due to reduced renal blood flow, micro-infarcts and interstitial fibrosis [20]. On radiological examination, PNH patients with renal involvement may exhibit enlarged kidneys, cortical infarcts, cortical thinning, and papillary necrosis.

Thrombosis in PNH

Thrombosis that immutably occurs in the venous system is the leading cause of mortality in PNH (40-67%) and 29-44% of patients with PNH have been reported to undergo at least one thromboembolic event during the course of their disease [21]. Patients who present with thrombosis have a 4-year survival rate of only 40% [22]. The abdominal and cerebral veins are the most commonly involved regions in venous thrombosis. Arterial thromboses can also be seen in PNH, mostly in the cerebral and coronary arteries.

The mechanism of thrombosis in PNH depends on many factors, including PNH clone size, hemolysis, platelet activation, inhibition of ADAMTS13 and impaired nitric oxide (NO) bioavailability.

There is a correlation between PNH neutrophil clone size and the occurrence of thrombosis, withan estimated odds ratio for thrombosis risk of 1.64 for every 10% increase in clone size. However, recent studies indicate that thrombosis can appear with smaller (10%) clones [23].

Free hemoglobin released from lysed PNH erythrocytes and uptake of monocyte-derived microparticles released from GPI-deficient monocytes upon complement damage (and which increase tissue factor [TF] expression) may be directly toxic to endothelial cells (EC). This is considered an important contributory factor in thrombosis [24].

PNH platelets undergo exocytosis of the MAC. Formation of micro vesicles with phosphatidylserine externalization is a potent *in vitro* procoagulant. Fibrinolysis is also perturbed, and PNH blood cells lack the GPI-anchored urokinase receptor. Tissue factor pathway inhibitor (TFPI) requires a GPI-anchored co-receptor for trafficking to the endothelial cell surface [25, 26].

Decreased NO levels may increase the risk of thrombosis in PNH through a number of mechanisms [27]. Free hemoglobin irreversibly reacts with NO to form nitrate and methemoglobin. Lysed erythrocytes release arginase to catalyze conversion of arginine to ornithine. Arginine is the substrate for NO synthesis. NO maintains smooth muscle cell relaxation, inhibits platelet activation and aggregation, and has anti-inflammatory effects on the endothelium.

Pregnancy and PNH

PNH occurs slightly more predominantly in females and can be seen in patients of childbearing age. It is difficult to manage pregnant PNH patients because complications of pregnancy can mimic PNH flares. The most common complication is the increased need for red blood cell and platelet transfusions. This is associated with higher maternal and fetal mortality and morbidity due to thrombotic events, infections, bleeding, anemia and prematurity [28]. The mortality rate in patients with PNH is reported as 6-20% during pregnancy and delivery [29].

The management of PNH in pregnancy depends on supportive treatment with washed blood products for symptomatic anemia and thrombocytopenia. Pregnant PNH patients have an

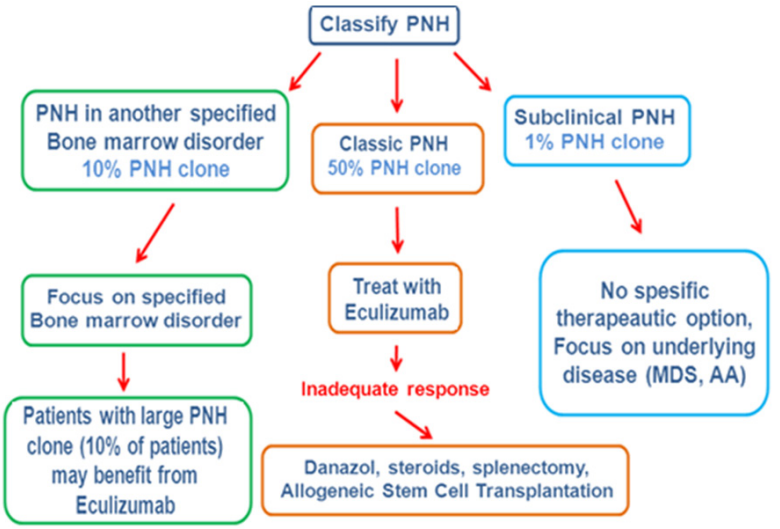

Figure 4. Treatment algorithm of PNH.

increased need for folic acid replacement due to hemolysis. There are no prospective trials concerning the role of anticoagulation inpregnant PNH patients so this issue remains controversial. Aside from anecdotal evidence from case reports, there are no published studies evaluating the use of eculizumab in pregnancy. Animal studies have shown that eculizumab can cross the placenta, and the drug has been listed as a a pregnancy category C agent [28].

Classification of PNH

Classification of PNH is necessary for general clinical management and decision-making with regard to treatment. In patients with classic PNH, leukocyte and platelet counts are usually normal, and the PNH clone is > 50%. These patients can present with multisystemic manifestations; bone marrow examination shows erythroid hyperplasia. Patients with subclinical disease have very small clone sizes (around 1%) that are not large enough to produce even biochemical evidence of hemolysis. Patients with PNH in the setting of another bone marrow failure syndrome (AA or MDS-RA) typically have a small clone (around 10%) and biochemical evidence suggesting mild hemolysis. Leukopenia and/or thrombocytopenia accompany PNH in this setting [11-14].

Treatment of PNH

Apart from bone marrow transplantation there is currently no cure for PNH. Interestingly. however, approximately 10-15% of patients show spontaneous remission, which may occur even after many years of the disease. Patients with subclinical PNH require no PNH-specific therapy. In patients with PNH in the setting of another bone marrow disorder the focus of treatment is on the bone marrow failure component of the disease [11]. The treatment strategy is generally developed for classic PNH (**Figure 4**).

Historical treatment

In the past, therapy was often restricted to the treatment and prevention of complications (e.g., red blood cell transfusions for anemia), but did not address ongoing hemolysis and its related symptoms; folate supplements were administered to support erythropoiesis and anticoagulation for the prevention of thrombosis [14]. Immunosuppression for the treatment of bone marrow failure and corticosteroid and androgen therapy have been used in PNH to help treat the anemia, but controlled data exist to question the clinical efficacy of this approach in terms of whether any potential benefits outweigh the established risks of such therapies [30].

Thrombolytic therapy can be recommended for patients with acute-onset abdominal vein thrombosis. However, in some patients, thrombolytic therapy or anticoagulation is contraindicated because of severe thrombocytopenia. Managing PNH patients on chronic warfarin therapy is often challenging. Platelet counts are usually mildly to moderately reduced. Maintaining a therapeutic international normalized ratio (INR) is difficult in some patients because of frequent PNH attacks that may be associated with anorexia, nausea, and vomiting. The use of oral contraceptives and pregnancy may exacerbate the proclivity for thrombosis in PNH, and should be considered high risk [31].

Targeted therapy with eculizumab

Recent laboratory investigations into a targeted therapeutic approach for PNH have emerged

in the last two decades, and in particular the availability of eculizumab makes it necessary to revise the conventional approach to the treatment of this disease. Eculizumab is a humanized monoclonal antibody that blocks the activation of terminal complement at C5 and prevents the formation of C5a and the terminal complement complex, C5b-9 [32]. The drug is delivered in an infusion for approximately 35 minutes, with a 600 mg induction phase of weekly infusions for the first 5 weeks followed by 900 mg every 2 weeks.

The impact and safety of eculizumab on the course of PNH was first established in a double-blind, placebo-controlled, 26-week Phase 3 trial (TRIUMPH) involving 87 PNH patients in 2006 [8]. Stabilization of hemoglobin levels in the absence of transfusions was achieved in 49% of patients. Eculizumab reduced intravascular hemolysis, as shown by an 85.8% lower median area under the LDH-time curve (in days) with eculizumab versus placebo ($p < 0.001$). Headache was the most common adverse event, there were no deaths during the study, and only a single thrombotic event occurred in a patient in the placebo group. Clinically significant improvements in QoL, as measured by scores on the Functional Assessment of Chronic Illness Therapy-Fatigue scale ($p < 0.001$) [8].

SHEPHERD, an open-label, 52-week Phase 3 trial involving 97 PNH patients, the second key trial designed to evaluate the safety and efficacy of eculizumabreported that 91.7% of patients maintained complete inhibition of serum hemolytic activity with every 14-day dosing throughout the duration of treatment, while only 8.2% of patients demonstrated a return of terminal complement activity and hemolysis during the last 1 or 2 days of the dosing interval. The mean number of packed RBC units transfused was reduced by 52% during treatment, and 51% of patients became transfusion independent. The most common adverse events were headache, nasopharyngitis, and upper respiratory tract infections, which were seen in 10% of patients. Two patients with a history of thrombosis experienced a thrombotic event. QoL was significantly improved in terms of fatigue and function scores [30]. Complete inhibition of serum hemolytic activity and thrombosis, and the improvement in QoL reported in SHEPHERD demonstrated that timing, regularity and continuity are important determinants for treatment outcomes during eculizumab therapy.

Hematopoietic stem cell transplantation

The only potentially curative treatment for PNH is allogeneic hematopoietic stem cell transplantation (HSCT). There are few reports on the use of HSCT for PNH that include small numbers of patients. Long-term cure rates as high as 60% have been reported with ASCT [7]. However, HSCT is not considered appropriate for all patients, and there are still some unanswered questions relating to the use of HSCT relating to the optimal timing of treatment, the best conditioning regimen, and the possible role for transplantation using an alternative donor.

QoL in PNH

The symptoms of PNH including fatigue, abdominal pain, dyspnea, dysphagia, chest pain and erectile dysfunction have a major adverse impact on patient QoL. It is therefore important to determine QoL even in patients with a small clone size. Treatment of PNH patients with eculizumab is associated with significant improvements in physical and social functioning of QoL [30].

Multidisciplinary organization: PNH education and study group

PNH has multiple symptoms and signs related to different organ sites, including the GI tract, kidneys, brain and musculoskeletal system. The first clinical visit of patients with PNH therefore varies widely based on the first sign or symptom. PNH patients can therefore present at neurology, gastroenterology, nephrology, general medicine, internal medicine, respiratory, cardiology and hematology clinics. However, it can take months to years for a patient to eventually visit the hematology unit and, because of this delay, patients often undergo persistant damage to affected tissues resulting in consistent organ failure.

In our experience it is necessary to adopt a multidisciplinary approach in order to increase the rate of early diagnosis. To address this challenge we have established a new group named the PNH Education and Study Group (PESG) in order to bring together scientists with an inter-

est in PNH. This group contains hematologists, immunologists, gastroeneteroligists, chest specialists, cardiologists, nephrologists and neurologists. The PESG is an open group for all medical disciplines with the major aim of increasing awareness of PNH and providing education to different medical specialities. Although it has only been established since 2013 it has already organized 12 educational activities in different regional areas, activities are organised per specific topic (cardiology, nephrology etc). The next aim is to extend PESG educational activities on a nationwide basis.

Conclusion

Contrary to common belief, PNH is not paroxysmal, not nocturnal and hemoglobinuria is seen less frequently. Although PNH has a special place in the field of hematology it is a multisystemic disease. Beyond hematology, other relevant disciplines including immunology, nephrology, gastroenterology, cardiology, pulmonary medicine and neurology should all be involved in the clinical management of PNH to provide a multidisciplinary approach.

Clinical trial evidence provides support for the central role of intravascular hemolysis in the pathogenesis of the disease, and indicates that eculizumab is an effective treatment in patients with PNH. In particular, the long-term administration of eculizumab substantially reduces the risk of thrombosis.

Disclosure of conflict of interest

The authors declare no competing financial interests.

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