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Development of the anti-Factor B iptacopan as single-agent treatment for paroxysmal nocturnal hemoglobinuria

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A. INTRODUCTION

1. PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

1.1 Definition and historical background.

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare acquired hematological disease of the hematopoietic stem cell, characterized by chronic complement-mediated intravascular hemolysis, thrombotic events and bone marrow failure. [1-4].

One of the first descriptions of the disease was made by Dr. Paul Strübing, who, in 1882, described a 29-year-old man who presented with fatigue, abdominal pain and severe episodes of hemoglobinuria. At that time, Dr Strübing already suggested a possible mechanism of intravascular hemolysis that, in the following years, was discovered to be the basis of the disease[5]. In 1911, Marchiafava and Micheli described the disorder in detail, coining the first definition of the disease as a "chronic hemolytic anemia with perpetual hemosiderinuria" [6,7]. However, it was Enneking, in 1925, who introduced the term "paroxysmal nocturnal hemoglobinuria" (PNH) [8]. In 1937, Thomas Ham observed that erythrocytes from PNH patients, if incubated with normal acidified serum, hemolyzed [9]. This fundamental discovery led to the realization of the first diagnostic test, which is still called by its name (Ham Test). Ham hypothesized that the lysis of cells incubated with acidified serum was complement-mediated, as heat led to the inactivation of the reaction. But since at that time only the antibody-mediated activation pathway was known, and hemolysis of PNH patients erythrocytes in acidic serum was an antibody-independent process, he could not prove the role of complement until the alternative pathway of the complement system was identified, in 1954 (by Pillemer and others) [10]. The 1980s led to a breakthrough in the understanding of the pathophysiology of PNH. Indeed, Dr. Parker and his team documented that the excessive susceptibility of red blood cells to complement-mediated hemolysis was due to the lack of some regulatory factors of the complement system expressed on normal erythrocytes, but missing on PNH erythrocytes. In particular, two proteins, the CD55 and CD59, regulators of the complement system, linked to the cell surface by glycosyl phosphatidyl-inositol (GPI) anchors, were identified. A few years later, the genetic mutation (phosphatidylinositol glycan class A [PIG-A]), responsible for the deficiency of the GPI anchor proteins, was identified; this gene codifies for an enzyme that acts in a fundamental step in the synthesis of GPI anchors, the molecular elements missing on the erythrocytes of these patients [11-14].

1.2 Etiology and molecular basis

PNH is caused by an acquired somatic mutation of the hematopoietic stem cell of the PIG-A gene, a housekeeping gene, located on the short arm of the X chromosome (Xp22.1) The PIG-A gene was first isolated in 1993 by Kinoshita, and then described in detail in 1994 by Bessel Bessler [14,21-24].

The PIG-A gene encodes an essential enzyme to transfer of N-acetyl-glucosamine to phosphatidyl-inositol. This is the first step necessary to form glycosyl-phosphatidyl-inositol (GPI), a glycopeptide used by the cells as an anchor system for many proteins physiologically located on cell surface. As a consequence of the mutation, the PNH cells are totally or partially deficient of the proteins anchored to the membrane by GPI ("GPI-anchored proteins", GPI-APs) [25-26.]

All mutations identified in PNH patients involve only the PIG-A gene; no abnormalities were found in the other 20 genes involved in the biosynthesis of GPI-APs.

Usually, each patient has only one mutation, even if rarely clones can coexist with different mutations. Most mutations of PIG-A gene are small insertions or deletions, usually 1 or 2 bp, that determine a frameshift mutation, with the consequent synthesis of product without function, leading to cells totally deficient of GPI. However, missense mutations may also be present, leading to the production of a minimal "amount" of GPI-APs[27].

Cells completely lacking in GPI-APs are defined PNH type III cells, while those still capable of producing small amount of GPI-APs are called type II; type I, on the other hand, defines cells with a normal GPI-APs expression profile.

These findings match the observation initially made by Rosse (Rosse 1966)[28,29], demonstrating the presence of distinct subpopulations of RBCs with different sensitivity to complement-mediated lysis.

It is possible to find, in the same patient, clones with type II and III mutations at the same time, thus revealing the possibility that mutated blood cells can expand simultaneously. This observation suggests that the clones with these mutations are selected on the basis of their GPI-deficient phenotype, due to a specific pathogenetic mechanism which implies the selection of GPI-deficient hematopoietic stem cells [29,30].

Small GPI negative clonal populations have also been documented in healthy individuals; from this observation it can be inferred that the mere presence of the genetic mutation is not sufficient to determine the pathology. Clonal expansion, starting from a single hematopoietic cell mutated, probably is due to the fact that the mutated cell is able to resist insults, probably of immune system, against which the normal cell is sensitive (see below) (figure 1).

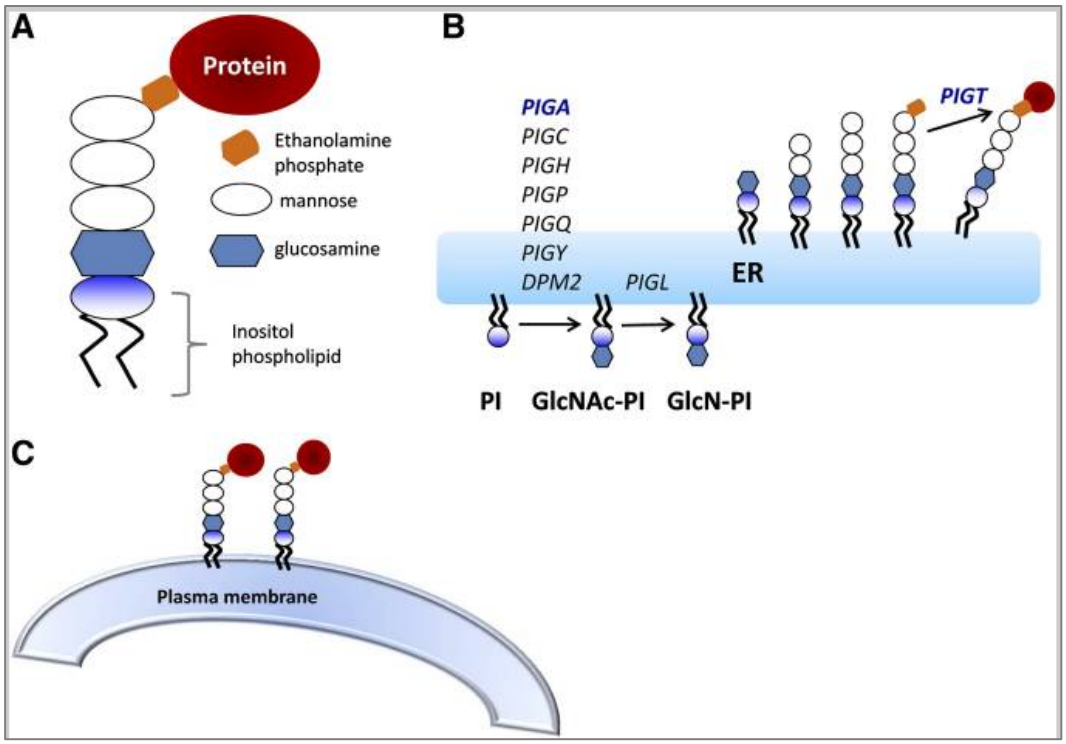


Figure 1. *Synthesis of the GPI anchor: In PNH cells the synthesis of the glycosylphosphatidinositol anchor is impaired, therefore all PNH cells do not express on their surface the proteins bound through the GPI anchor. (Brodsky, Blood 2014)*

1.3 Pathogenesis

Among the GPI-anchored proteins, CD59/MIRL (Membrane Inhibitor of Reactive Lysis) and CD55/DAF (Decay-Accelerating Factor), two regulators of the complement system, play a key role in the pathogenesis of PNH [31-34]. CD55, a 68,000 kilodalton (KDa) glycoprotein, controls early complement activation, inhibiting the C3 and C5 convertase; while CD59, a 19,000 KDa glycoprotein, interferes with the terminal phase of the complement system, blocking the incorporation of C9 into the C5b-C8 complex and blocking the formation of the membrane attack complex (MAC)[35-38]. The lack of CD55 and CD59 exposes PNH red blood cells (RBCs) to complement-mediated lysis, with consequent chronic intravascular hemolysis. Furthermore, there is a constant minimal activity of the complement system caused by "tick-over phenomenon" of the alternative pathway, due to a minimal spontaneous hydrolysis of C3 (while the other two pathways, classical and lectin, require specific stimuli to be activated). In addition to this chronic continuous, steady-state low-grade hemolytic activity, possible hemolytic crises (the so called "paroxysms") may occur, in concomitance with specific stimuli able to amplify the activity of the complement activity (such as inflammatory and / or infectious pathology) [39,40].

CD59 seems to have a predominant role, as rarely evidenced in patients with hereditary deficiency of a single molecule: while the only patient described with a hereditary deficiency of CD59 had clinical manifestations similar to PNH, the CD55-deficient subjects, a relatively common condition called the Inab phenotype, show no signs of hemolysis.
It is not yet completely clear why the exacerbation of the hemolytic crises would occur at night; typical nocturnal crises that gave the name to the disease. However, the reduction of the blood pH due to the retention of CO2 already identified by Ham may be involved (the increased concentration of first morning urine should also be considered).

CD55 and CD59 are also absent on the surface of PNH white blood cells (WBC) and platelets (PLT). However, there is no evidence of a reduction in the half-life of PNH WBCs, probably due to the presence of additional molecules, non-GPI-linked (CD46), or to other mechanisms that protect these cells from complement-mediated lysis [34,44]. Instead, the activation of the complement system on the platelet surface has been proposed as a possible cause of thrombotic episodes, characteristic in PNH patients. It has been hypothesized that the activation of the complement would determine the activation and subsequent platelet aggregation; even if no scientific evidence of this mechanism has been provided yet. However, further hypotheses have been proposed to explain thrombophilia in PNH patients; one cause, for example, could be an alteration of the fibrinolytic system due to the absence on the surface of PNH neutrophils of the uPAR urokinase receptor (CD87), a GPI-linked molecule, whose soluble form exceed in the plasma of PNH patients, reducing plasmin formation. The development of thrombotic episodes could also be directly due to intravascular hemolysis, as a consequence of the increase in plasma of free hemoglobin that binds and reduces nitric oxide (NO) concentrations; in this way, the anti-aggregating, anti-adhesive and vasodilating effect characteristic of NO would be lost. Obviously, other inherited or acquired risk factors can increase the predisposition to the development of thrombotic events, such as the factor V Leiden mutation or mutations in the MTHFR gene, resulting in hyperhomocyteinemia [27,40,45-50].

Mutation of the PIG-A gene is the hallmark of the disease, but this mutation while necessary is not sufficient to cause expression of the clinical phenotype, as suggested by the following observations:

- ✓ 10-50 granulocytes per million expressing the PNH phenotype can also be found in normal individuals, as demonstrated by flow cytometry and PCR that identifies the mutation [51,52];
- ✓ Lymphocytes deficient for GPI molecules have been found in patients with lymphoma immediately after treatment with alemtuzumab (a humanized monoclonal antibody (mAB) that recognizes the CD52 molecule, GPI-linked); they progressively disappear after treatment discontinuation [53];
- ✓ PNH population expansion is difficult to reproduce in mouse models; a knock-out animal cannot be generated, as the embryonic mutation of the PIG-A gene is lethal. At least in these models, it appears that the PNH stem cell has no proliferative advantage over the normal stem cell; this is also confirmed by in vitro growth experiments in which PNH patient cells and wild-type hematopoietic cells were used [54-57].
- ✓ PNH hematopoiesis is certainly clonal but not necessarily monoclonal.

Indeed, in some cases PNH hematopoiesis is supported by more than one mutated (oligoclonal) clone, as demonstrated by the different susceptibility to complement-mediated lysis of the different sub-clones, or as evidenced by flow cytometry. This expansion of cellular elements with the same functional defect, but which are molecularly heterogeneous, could be compatible either with a random process or with a process of selection of the stem cell with PNH phenotype over the normal stem cell [28,58-59].

All of this evidence led to the formulation of the dual pathophysiology of PNH, also known as "relative advantage" or "escape theory" [3,4,61].

According to this theory, a mutation of the PIG-A gene may be a relatively common event in the context of normal hematopoiesis, which remains without clinical consequences since the PNH stem cell does not have an intrinsic proliferative advantage over the normal stem cell [62]. External factors can alter this status, creating a permissive environment for the expansion of the PNH stem cell, which can, therefore, support hematopoiesis even for the patient's life [63]. The nature of this external event is probably immunological, as suggested by the fact that some PNH patients have impaired bone marrow function up to severe aplasia. It is likely that this pathogenetic mechanism, which is also the basis of the etiopathogenesis of aplastic anemia, may play a key role in PNH, representing the additional factor necessary for the development of “clinical PNH”, in patients who already present the mutation of the PIG-A gene. In acquired bone marrow failure syndromes, the "normal" hematopoietic stem cells are exposed to an attack, probably of immunological origin, whose the target has not yet been identified, nor has

the mechanism that determines the loss of tolerance been understood; this leads to an complete destruction of the hematopoietic compartment and consequent cytopenia. Unknown triggers determine a cellular immune response, which results in an expansion of T lymphocytes clones, leading to the destruction of the stem cell, either directly through the action of cytotoxic T lymphocytes (CTLs), or indirectly through the production of inhibitory cytokines, such as interferon gamma or TNF-alpha [64-66].

It is reasonable to assume that a stem cell may occasionally and randomly acquire a mutation of the PIG-A gene, but this does not result in disease expression because of the lack of intrinsic proliferative advantage of the PNH stem cell over the normal one, unless a second event, in this case an immunological attack, occurs against the hematopoietic stem cell. This attack, probably directed against a GPI-linked antigen (or the GPI anchor itself), would lead to the destruction of the stem cell compartment that assures normal hematopoiesis. As a consequence of the depletion of the physiological compartment, the PNH stem cell, preserved from immunological attack, can expand. Therefore, the PNH stem cell expansion is proposed as an "escape" mechanism against the immunological attack directed at the hematopoietic stem cell and an exclusive consequence of the selective negative pressure on normal stem cells. Indeed, il can also be argue that the expansion of a PNH clone (and thus the emergence of a clinical PNH) may be considered as a self-cure for an immune-mediated bone marrow failure, which would alternatively lead to a severe aplastic anemia (figure 2).

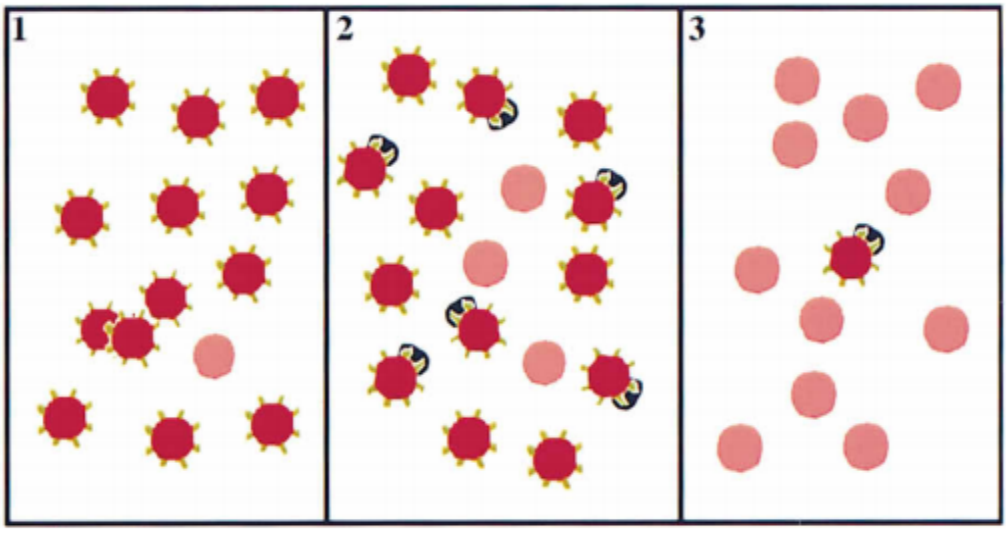


Figure 2. Dual physiopathology theory (Rotoli, Luzzatto 1989)

In this image, normal hematopoietic cells (HSCs) are represented in dark red, which present on their surface protrusion, representing GPI-anchored proteins (GPI-APs). In pink, on the other hand, the HSCs that have acquired a mutation in the PIG-A gene, and, for this reason, are missing GPI-APs. Black symbols represent "hypothetical" molecules that, by binding the GPI-APs, lead to the destruction of the HSCs.

Panel 1: a normal bone marrow is shown, in which some mutated PIG-A cells can coexist.
Panel 2: Normal HSCs undergo an attack that targets GPI-APs. The mutated HCs-PIG-A are not affected by the attack. Hematopoiesis becomes a mosaic of normal and PNH cells.
Panel 3: Persistence of negative selective pressure on normal HSCs leads to the relative expansion of mutated HCs-PIG-A; thus, hematopoiesis becomes predominantly PNH phenotype.

1.4 Epidemiology

PNH is a rare disease, with incidence of 1.5-2 cases per million of the population per year and with a prevalence of 15.9 cases per million of the population, similar to aplastic anemia (AA) [67-69]. Some authors indicate that this number is probably low, as the disease remains undiagnosed in individuals with limited symptomatology, or with comorbid conditions that obscure the PNH diagnosis [70].

PNH can affect patients of any age, although it is frequently observed in young adults. Women are affected at a slightly higher rate than men; as the PIG-A gene is on the X-chromosome, the probability of developing the disease is almost the same regardless of sex, with the mutation occurring in somatic hematopoietic cells in which the active X- chromosome is always only one, even in women, due to lyonization. There is no hereditary transmission because the mutation does not occur in the germ cells.

1.5 Clinical features e natural history

The peripheral blood of PNH patients is a mosaic of normal and pathological cells. The degree of mosaicism is determined by the level of expansion of the mutated clone(s), but the factors determining the degree of clonal expansion are still not completely understood, although several hypotheses have been postulated [3, 23, 71, 72]. Although PNH is a clonal disorder, it is not a malignant disease and, for unknown reasons, the size of expansion of the PIG-A clone is highly variable in different patients. In some cases, more than 90% of peripheral blood cells may derive from the clone, whereas, in other cases, less than 10% of circulating cells have a defect in the expression of GPI-APs. The variability in the degree of mosaicism is clinically relevant, as patients with small PNH clones have few or no symptoms and do not require specific treatments, while patients with large clones often present important symptoms as a consequence of chronic massive intravascular complement-mediated hemolysis and require specific therapies with complement inhibitors.

Clinically, PNH is characterized by a typical triad: hemolytic anemia with related signs and symptoms, propensity to thromboembolic events and bone marrow failure.

Based on the different clinical presentations, the following classification of the disease, proposed by the International PNH Interest Group [73], is widely used today (figure 3):

- ✓ Classical PNH, in which hemolytic manifestations without bone marrow dysfunction predominate;
- ✓ PNH in the context of other pathology with bone marrow failure (e.g., aplastic anemia, myelodysplastic syndromes, or myelofibrosis)
- ✓ Subclinical PNH, characterized by the presence of PNH clones in the absence of clinical evidence or laboratory findings suggesting hemolysis, in the context of another bone marrow disorder (e.g., AA or myelodysplasia).

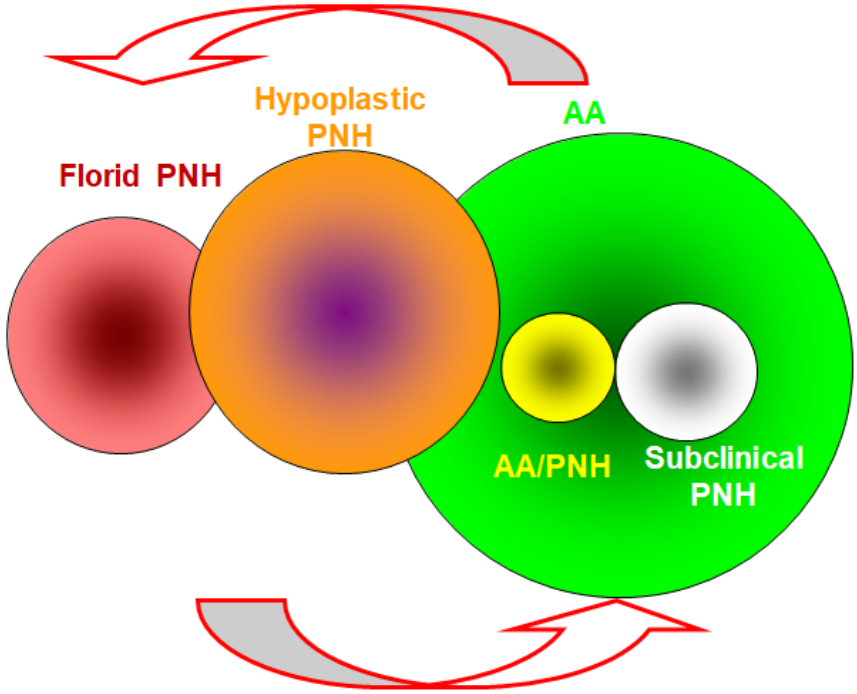


Figure 3. Clinical overlap between aplastic anemia and PNH (A.M. Risitano)

This classification, however, has limitations in its use, because, often, the clinical presentation can fall into more than one described category. In fact, a certain degree of bone marrow failure is present in all PNH patients, in accordance with the pathophysiological mechanisms described above. In fact, patients with severe bone marrow failure can present different degree of hemolysis, overt, typical of the classical form, or more blurred, typical of the intermediate form. Therefore, establishing precise cut-offs is extremely complex, and usually clinically irrelevant. The main clinical manifestation of PNH is certainly hemolysis; indeed, looking at the disease's name, one should argue that there is no PNH without hemolysis. It is manifested with different

severity, depending on the size of the PNH clones, the sensitivity to complement-mediated lysis of PNH RBCs (Type II or Type III), and the degree of the complement activation (which is widely variable over time, depending on medical conditions or individual patient characteristics). Hemolysis is typically chronic, due to the minimal spontaneous activation of the complement system (C3 tick-over), already mentioned, with paroxysms related to trigger events generally consisting in infections or other acute inflammatory events. The intravascular hemolysis leads to a range of symptoms and signs; the most remarkable is hemoglobinuria, resulting from the passage of hemoglobin released from lysed erythrocytes in the urine, which causes the typical dark color. Depending on the urinary concentration, the color can range from a dark yellow or dark red to black (typical of the first morning urine). The color of urine is often described as marsala (by Dr. Marchiafava in his pioneering description of PNH) or Coke colored.

The resulting anemia can be variable and multifactorial. In fact, it depends on the degree of complement activation, on the capacity of residual erythropoiesis (depending on the level of bone marrow failure, but also on any deficient conditions, for example due to chronic iron loss through hemosiderinuria). For this reason, some patients have normal hemoglobin levels or at least within normal limits, while others present a severe anemic state requiring intensive transfusion support.

Typical signs of intravascular hemolysis are jaundice, hemoglobinuria, increased LDH and indirect bilirubin values, decreased haptoglobin concentration, presence of hemosiderin in the urinary sediment and variable reticulocytosis, depending on the degree of underlying bone marrow failure. Other possible accompanying clinical manifestations are abdominal pain, dysphagia, esophageal spasm, pulmonary hypertension, due to the binding of free hemoglobin with nitric oxide resulting in contraction of the vascular and gastrointestinal smooth muscles. Frequent symptoms, closely related to the degree of anemia, are asthenia, fatigue, lethargy, and significant reduction in the quality of life [49,74].

Another typical manifestation of PNH are thromboembolic events, which are the main cause of morbidity and mortality in this pathology. The propensity to venous thromboembolic events is characterized by the involvement of atypical sites, such as the hepatic veins (Budd-Chiari syndrome), mesenteric veins, splenic vein, cerebral venous circulation (sagittal and cavernous sinus). The incidence of thrombosis appears to be variable in the population, with a reduced rate in Southeast Asia compared with Europe and the United States. This could be related to the

complex pathophysiology of these events, as described previously, and to the possible contribution of various genetic predisposing factors [75,76].
Several observations have reported massive intravascular hemolytic crises and large EPN clones on WBCs as predisposing factors for thrombosis. However, even patients with a small PNH clone have an increased thrombotic risk compared to healthy individuals [76].

Finally, in several PNH patients cytopenia may be present, depending on the degree of underlying bone marrow failure, which may be moderate to severe to leading of the development of severe aplastic anemia, requiring specific treatment. The clinical manifestations of cytopenia depend on the line involved and the severity of the presentation. Patients may present an increased risk of bleeding, depending on the reduced concentration of platelets, and/or an increased incidence of infectious events, depending both on the reduced number of WBCs, but, probably, also on their reduced functional capacity [77].
The natural history of PNH is not predictable because of its heterogeneity in presentation and clinical course. The median survival, after diagnosis, of an untreated individual is 10 years, with about 25% of patients achieving a survival of about 22 years. An estimated 2-3% progression to acute leukemia is reported, similar to what happens in patients with aplastic anemia. The main causes of death in PNH patients are thrombotic events (especially cerebral thrombosis 25% and S. di Budd-Chiari 23%) and infectious complications (25%) [78-82].

1.6 Diagnosis

The diagnosis of PNH remains a clinical diagnosis and should be suspected in patients with moderate-severe anemia with reticulocytosis, elevated LDH levels, moderate jaundice, and negative Coombs test. In addition, the presence of dark urine (Coke or marsala colored) (figure 4), of hemosiderin in the urinary sediment, of a variable degree of leuko-thrombocytopenia and, finally, of a positive anamnesis for thromboembolic events in atypical venous sites is strongly suggestive of PNH. In particular conditions, PNH can be suspected even in the absence of clinically evident hemolytic anemia such as in patients with AA or with recurrent thromboembolic events in the absence of risk factors.

Figure 4. *Hemoglobinuria: several urine samples are illustrated, the first three frankly hemoglobinuric, Coca-Cola color, the others with different degrees of hemoglobinuria.*

The diagnosis requires the documented partial or total absence of GPI-APs on the surface of at least two blood cell lines. The absence of the clone does not allow a diagnosis of PNH to be made; on the other hand, its presence must be evaluated in the patient's clinical context [83-84].

For years the "gold standard" for diagnosis has been the Ham Test, also referred to as an acidified serum test. It consists in incubating the patient's red blood cells in a compatible serum, but acidified with HCl, in such a way as to activate the alternative complement pathway. Lysis does not occur if the same serum is brought to a temperature of 56 ° C, a temperature that causes the inactivation of some components of the complement, or when erythrocytes of healthy individuals are tested [85]. Although simple to perform, this test has several limitations; in fact, besides being technically difficult to standardize and requiring time and experience for its execution, it is not very sensitive, because it is difficult to detect the hemolysis when it pertains a proportion of red blood cells of less than 5%. Furthermore, with this test it is not possible to discriminate PNH type III, with total GPI-APs deficiency, from PNH type II, with partial deficiency. Ham's test has now only historical importance [86].

Since 1996, the accepted method for the detection of PNH clone is flow cytometry, which, through the use of monoclonal antibodies conjugated to fluorochromes directed against the single GPI-APs, allows to discriminate cells with total (type III) or partial (type II) defect expression of these proteins. The normal cellular component is classified as type I. It also allows to determine the size of the clone expressed as a percentage of cells showing a deficit of expression of GPI-APs and to follow its trend over time. Finally, the sophisticated techniques available allows the detection of even small clones. The cell lines analyzed are neutrophil granulocytes, for the identification and correct quantification of the clone, the erythrocytes, mainly for clone detection, as the size may be underestimated due to hemolysis and/or transfusions, and monocytes, to confirm the presence of the clone. Clone size is determined by the percentage of neutrophil granulocytes that do not express GPI-APs [87]. Various monoclonal

antibody panels have been proposed for this test, usually including CD55 and CD59 for erythrocytes, CD66b, CD66c and CD24 for granulocytes, CD14 and CD48 for monocytes, and CD48 or CD59 for lymphocytes. Platelets are not normally tested for PNH phenotype due to the difficulty in being separated from normal platelets due to their small size [87-90]. For the analysis of the leukocyte component the reference reagent is fluorescent aerolysin (FLAER), an inactivated bacterial toxin of 52 kDa able to bind to the GPI anchor and therefore capable of identifying the presence or absence of all "GPI-linked" molecules. The analysis is to be performed on peripheral blood anticoagulated with EDTA or heparin, preferably within 24-48 hours after collection, in order to prevent problems related to cell mortality.

Finally, molecular studies on DNA or mRNA, aimed at identifying the mutation in the PIG-A gene, have no clinical relevance, in fact small clones of PNH cells can also be found in healthy subjects: therefore, it is important to identify the clone, but even more is to evaluate its presence within a defined clinical scenario.

1.7 Treatment: the pre-Eculizumab era

PNH treatment was mainly supportive until 2005, to control the typical manifestations of the disease. The main problems to manage are hemolysis and the consequent anemia, thrombotic events and bone marrow failure. Concerning hemolysis and subsequent anemia, the therapeutic options were often unsatisfactory. Steroids have been widely used both in chronic and acute settings in concomitance with hemolytic crises [91-,92]. Probably, steroids seem to give a benefit on symptoms related to hemolytic crises, such as dysphagia, abdominal pain, but they do not block the activation of complement system and therefore hemolysis . Their use, at a dosage of 0.5-1.0 mg/kg, correlates with significant long-term toxicity without a clear benefit on the pathology, and is strongly discouraged by all PNH experts. Androgens have also been used to stimulate erythropoiesis and megakaryopoiesis, with not so many benefits on hemolysis, but efficient on bone marrow function, especially in conditions with bone marrow failure associated [93,94]. However, the risk-benefit ratio must be adapted to the patient's clinical condition, taking into account side effects, such as liver toxicity, virilization, and, not to forget, the increased risk of developing Budd-Chiari syndrome [93]. Some patients have shown clinical benefit with the use of danazol given at a dosage of 400-600 mg/day.

Considering the impossibility of blocking the mechanism of intravascular hemolysis, supportive therapy with red blood cells remains the first choice to increase hemoglobin values, which should be maintained above 8 g/dl. This leads to an improvement in general symptoms, as higher hemoglobin levels reduce the erythropoiesis stimulation with less PNH cells

production. In contrast to polytransfused patients, in PNH patients, given the persistent hemosiderinuria, an iron deficiency is often present, so iron supplementation can increase hemoglobin levels in many cases (although sometimes the improvement in bone marrow production causes an increased hemolysis, because most of the red blood cells produced have the PNH phenotype and are susceptible to complement-mediated hemolysis); in the same way, vitamin B12 and folate supplements are often indicated to promote compensatory erythropoiesis secondary to hemolysis. Finally, the use of recombinant erythropoietin may be helpful, if the endogenous levels are inadequate [92,96].

The treatment of thrombophilia represents one of the most debated points in the therapy of PNH, because it represents the first cause of death for this pathology. However, there are no studies concerning the efficacy and therefore the indication for a primary or secondary antithrombotic prophylaxis, as well as discussed is the treatment of acute thrombotic event. Regarding primary prophylaxis, some experts recommend the use of warfarin for newly diagnosed patients, while others believe it is not necessary. Both approaches can be considered feasible, given the unpredictability of thromboembolic events and the lack of studies supporting these therapeutic strategies; the possible benefits must be balanced against the bleeding risk, given by warfarin therapy, in patients with low platelet count. If necessary, it is reasonable to proceed with primary prophylaxis in those patients who have additional risk factors, (such as Leiden factor V, etc.). About secondary prophylaxis, there is a greater agreement to subject patients who have already had a thrombotic episode to anticoagulant therapy for life, although there are also different opinions about the best strategy. Many authors prefer to start from low molecular weight heparin and then move on to warfarin. Despite the different strategies that can be applied, as mentioned above, the recurrence rate of a thrombotic episode in PNH patients is still very high [97,98]. In the treatment of an acute thrombotic episode, anticoagulant drugs have been used at therapeutic doses similar to the treatment for other thrombosis [99]; good results have been observed in some cases with fibrinolytic therapy.
In any case, as in anemia, the introduction of Eculizumab has substantially changed the management of thrombophilia of PNH.

Concerning the treatment of bone marrow failure, both supportive strategies with anti-infectious, anti-thrombotic and anti-hemorrhagic prophylaxis, and also etiological therapies based on different immunosuppressive regimens can be attempted. According to the pathogenetic mechanisms described above, an immune-mediated inhibition of hematopoiesis is

also hypothesized in PNH, similar to what happens in aplastic anemia. For this reason, immunosuppressive strategies have been tried in PNH patients: in some minor studies cyclosporine A showed some benefit, while other groups tried more intensive regimens using anti-thymocyte globulin combined with high-dose prednisone and cyclosporine A, showing heterogenous results.

Alternative regimens based on cyclophosphamide or the anti-CD52 monoclonal antibody Alemtuzumab could be an alternative as salvage therapy [100-102].

In any case, currently aplasia anemia represents the main indication for stem cell transplantation in these patients.

The only curative strategy for PNH patients is hematopoietic stem cells transplantation (HSCT). It has been attempted since the 1980s and has shown to be effective in eradicating the PNH clone, leading to recovery from the disease, although a high rate of early mortality related to the procedure. Most of the cases reported in the literature refer to single cases or to small numbers of patients generally from the same institutions [103-105], large-scale prospective studies have been lacking. A series was reported by Parker et al in 2005, in which 67 HSCTs were performed in PNH patients, transplanted from different donor types (syngeneic, sibling or HLA-identical unrelated) and with different conditioning regimens (myeloablative or reduced intensity), showing a long-term survival of 75%.[104].

Another retrospective study by GITMO (Italian Group Bone Marrow Transplantation) on 23 PNH patients transplanted between 1998 and 2006 reported an overall survival rate of 70%, with a median follow-up of 107 months [106]. In light of these results and the lack of studies on larger cohorts, it is difficult to identify guidelines for HSCT in PNH patients. At present, the main indication is the concomitant presence of bone marrow failure, and, as with aplastic anemia, HSCT should be considered first-line treatment when HLA-identical sibling donors are present, or in case of therapeutic failure in patients with an unrelated but HLA-matched donor [107-110]. While in the past refractoriness to transfusions and repeated severe thrombotic episodes were also indications for transplantation, today these conditions represent more an indication for anti-complement treatment than for transplantation; although the latter remains a good second-line choice for those who do not present an adequate response to eculizumab.

2. ANTI-COMPLEMENT TREATEMENT FOR PNH

2.1 The "Eculizumab" era

Eculizumab (Soliris ®, Alexion Pharmaceuticals) is a humanized monoclonal antibody, which binds the C5 fraction of the complement system, preventing its cleavage into C5a and C5b, thus

blocking the formation of the membrane attack complex (MAC), which is the terminal effector mechanism causing intravascular hemolysis of PNH erythrocytes [111] (figure 5). In addition, Eculizumab prevents the release of pro-inflammatory mediators, resulting from the cleavage of C5a. Blocking the complement cascade at the level of C5, however, does not affect the functioning of the proximal portion of the system (of C3 and its cleavage products), preserving the clearance function of immune complexes and microorganisms [112].

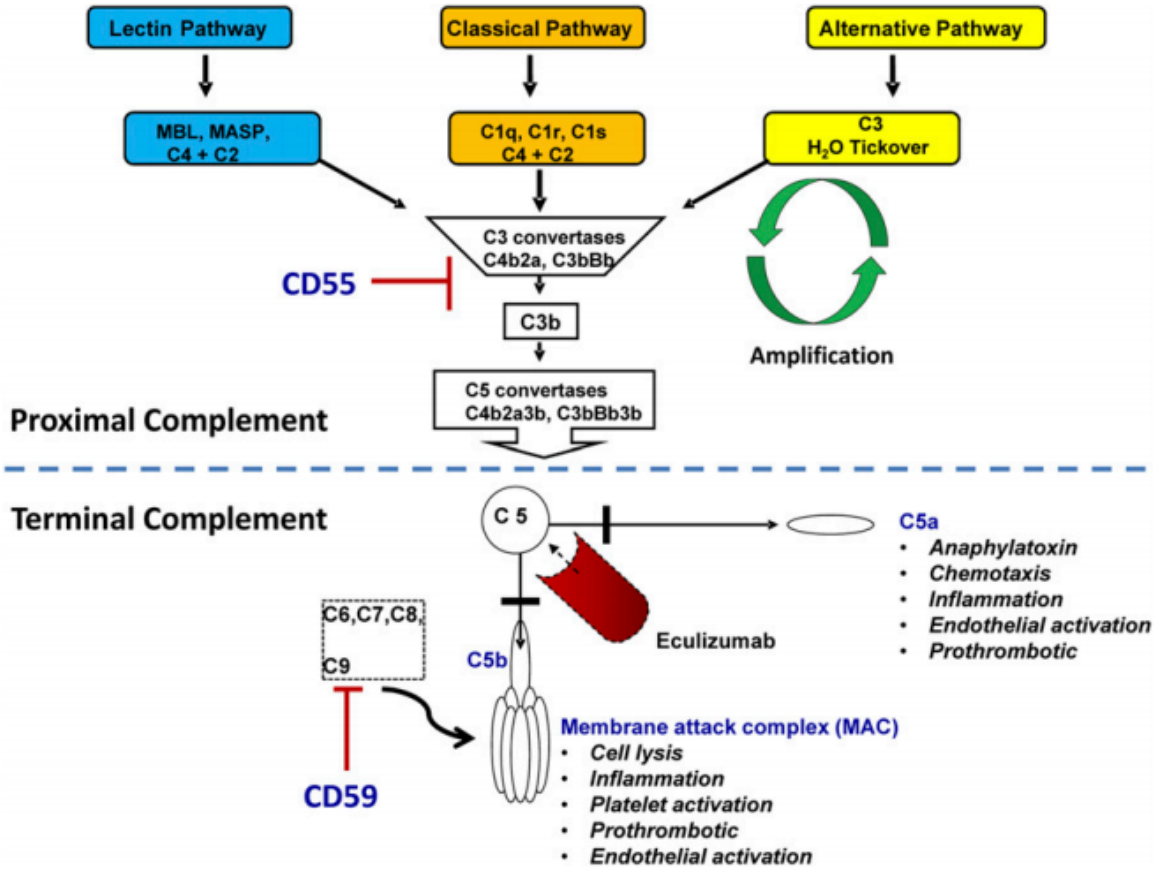


Figure 5. *Complement cascade and mechanism of action of Eculizumab (Brodsky, Blood 2014)*

Eculizumab was initially developed for patients with rheumatoid arthritis, psoriasis and systemic lupus erythematosus, however, in PNH it has found its best application. In fact, the lack of CD59 on PNH RBCs determines an uncontrolled activation of the MAC, with consequent chronic intravascular hemolysis; Eculizumab "compensates" for this absence by preventing its formation and protecting the PNH RBCs.

Eculizumab is administered intravenously and the treatment regimen, approved in 2007 by the American and European regulatory bodies (FDA and EMA), includes an initial phase consisting

of four weeks at doses of 600mg, followed by a dose of 900mg every two weeks, starting from the fifth week. Patients on eculizumab are at risk of meningococcal infections. Treatment should be preceded by a minimum of 15 to 21 days of tetravalent conjugate meningococcal serotypes ACWY135 and meningococcal B vaccine.

In case of an emergency start to treatment with eculizumab, especially in situations of thrombosis threatening the prognosis, effective treatment of meningococcal infection must be started (by beta-lactam or quinolone) and continued until 14 days after the first vaccines are administered.

The efficacy and safety of this drug have been widely demonstrated by a series of pivotal clinical trials. The first pilot study, in which 11 patients were enrolled, demonstrated effective inhibition of the complement system, at the C5 level, leading to a reduction in hemolysis in PNH patients, who presented a high transfusion requirement [113,114]. Subsequently, definitive evidence of efficacy has been proven by two multicenter studies, TRIUMPH and SHEPERD.

The TRIUMPH, a randomized double-blind, multicenter study, enrolled 87 patients available to receive Eculizumab or placebo [115]. This study demonstrated the efficacy of Eculizumab to reduce intravascular hemolysis (measured by lactate dehydrogenase (LDH) levels), with a stabilization of hemoglobin levels and independence from transfusions in about half of the treated patients, being also effective in reducing general symptoms of the disease, compared with placebo. These data were further confirmed by the SHEPERD trial, an open-label study, designed to test eculizumab in a larger population of PNH patients, also including patients with minimal transfusion requirements and thrombocytopenia [116]. In this study, a total of 97 patients were enrolled and received treatment for 52 weeks. Among the beneficial effects of the drug were demonstrated: a reduction in hemolysis, with a reduction in LDH levels of 86% (from an average of 2051 U/L to 297 U/L after 12 months of treatment); the elimination of all symptoms related to hemolysis, the reduction of transfusion needs, with at least half of the patients becoming transfusion-independent, and with a reduction in transfusion requirements in the other half.

Another aspect evaluated by P. Hillmen et al in 2007 [117], was the incidence of thromboembolic events (TE) after eculizumab treatment. Clinical trial participants included all patients in the 3 eculizumab PNH clinical studies, which recruited patients between 2002 and 2005 (n=195). Thromboembolism rate with eculizumab treatment was compared with the pretreatment rate in the same patients. The TE event rate with eculizumab treatment was 1.07 events/100 patient-years compared with 7.37 events/100 patient-years ($P < .001$) prior to eculizumab treatment (relative reduction, 85%; absolute reduction, 6.3 TE events/100 patient-years). With

equalization of the duration of exposure before and during treatment for each patient, TE events were reduced from 39 events before eculizumab to 3 events during eculizumab (P < .001). The TE event rate in antithrombotic-treated patients (n=103) was reduced from 10.61 to 0.62 events/100 patient-years with eculizumab treatment (P < .001). These results show that eculizumab treatment reduces the risk of clinical thromboembolism in patients with PNH.

It is not known whether this reduction is attributable only to an indirect action related to the inhibition of hemolysis or to a specific effect with a real reduction in the patient's thrombophilic condition, an, in this sense, a significant plasma reduction of the markers of coagulation activation, reactive fibrinolysis and activation of endothelial cell has been reported in patients on eculizumab[118]. This finding, once again, suggests a multifactorial pathogenesis of thrombosis in these patients, with a possible involvement of multiple metabolic pathways.

All these beneficial effects translate globally into a large increase in the long-term survival of patients treated with Eculizumab, which, on the basis of British data, is 90% at 9 years (well above historical data in the pre-eculizumab era) [119] and rates of thrombosis reduced from 5.6 events to 0.8 events per 100 patient-years with Eculizumab treatment [120].

Eculizumab does not have any significant side effects and it is well tolerated [121].

Eculizumab is an expensive drug and must be administered for the patient's entire life in order to maintain a lasting response; therefore, patients with blurred symptoms should be followed over time with a “watch and wait” approach. Conditions of severe anemia, thrombotic events, painful paroxysmal crises, asthenia, worsening of renal function are an absolute indication to start treatment [84].

2.1.1 Response categories and unmet clinical needs for PNH patients in the eculizumab era.

Response to eculizumab can be extremely heterogeneous among patients, and different response categories can be identified. Already in 2009, Risitano et al [122], proposed a first empirical classification of the hematological response in patients treated with eculizumab: optimal response (no transfusion, hemoglobin > 11 grams per deciliter (g/dL)), good response (no transfusion, hemoglobin between eight and 11 g/dL), partial response (transfusion requirement present, but reduced by at least 50% compared with the baseline), and minor response (transfusion requirement unchanged, or reduced by less than 50%). From this first categorization it emerged that only one-third of patients treated with eculizumab reach normal hemoglobin levels.

Approximately 10 years later, additional data confirmed that eculizumab treatment is effective and safe, but nevertheless leads to rather variable hematological responses [19,121,123]. Therefore,

in addition to transfusion independence, the hemoglobin value seems to be the most appropriate endpoint on which to evaluate the hematological response. In fact, considering that often today patients arrive at treatment with eculizumab before receiving transfusions, we have recently proposed a new classification of hematological response to anti-complement therapies based on hemoglobin and red blood cell transfusion, by adding markers of hemolysis (LDH) and absolute reticulocyte count (ARC). Six distinct biological categories have been determined: complete response (no transfusion, normal hemoglobin values and no sign of hemolysis, assessed with LDH and reticulocytes); major response (no transfusion, normal hemoglobin values, but signs of hemolysis); good response (no transfusion, hemoglobin values between 10 and 12 g/dL and evidence of intravascular hemolysis residual); partial response (hemoglobin values between eight and 10 g/dL, occasional transfusion support); minor response) [124].
(Figure 6).

Response category	Red blood cell transfusions	Hemoglobin level	LDH level*‡	ARC*
Complete response	None	≥12 g/dL	≤1.5x ULN	***and*** ≤150,000/μL§
Major response	None	≥12 g/dL	>1.5x ULN	***or*** >150,000/μL§
Good response	None	≥10 and <12 g/dL	A. ≤1.5x ULN B. >1.5x ULN	Rule out bone marrow failure°
Partial response	None or occasional (≤2 every 6 months)	≥8 and <10 g/dL	A. ≤1.5x ULN B. >1.5x ULN	Rule out bone marrow failure°
Minor response#	None or occasional (≤2 every 6 months) Regular (3–6 every 6 months) Reduction by ≥50%^	<8 g/dL <10 g/dL <10 g/dL	A. ≤1.5x ULN B. >1.5x ULN	Rule out bone marrow failure°
No response#	Regular (>6 every 6 months)	<10 g/dL	A. ≤1.5x ULN B. >1.5x ULN	Rule out bone marrow failure°

*LDH, lactate dehydrogenase; ULN, upper limit of the normal; ARC: absolute reticulocyte count. *Response categories are mostly based on red blood cell transfusion and hemoglobin level, but LDH and ARC serve as ancillary indicators to discriminate between complete and major response, as well as within suboptimal response categories. ‡A. and B. indicate subcategories without or with residual significant intravascular hemolysis, respectively. §To rule out increased erythropoietic response to compensate ongoing hemolysis; the value of 150,000/μL is a tentative index based on 1.5x ULN (which in most laboratories is set at 100,000/μL). °To assess the relative contribution of the degree of bone marrow failure to any response less than complete: a value of ARC below 60,000/μl could be a tentative index to establish such a contribution; bone marrow investigation may be appropriate. ^For patients with previous transfusion history (with a pre-treatment follow up of at least 6 months). #For patients who do not accept red blood cell transfusions, minor response can be defined based on hemoglobin level ≥6 and <8 g/dL, and no response based on hemoglobin <6 g/dL. All hemoglobin, LDH and ARC values should be assessed based on the median value over a period of 6 months.*

Figure 6. *Response categories (Risitano et al, 2019)*

We have also retrospectively applied a new classification to assess hematological responses in a large cohort of 160 PNH patients treated with eculizumab in six international PNH reference centers [125]. Our classification is based on hemoglobin level and red blood cell transfusion, by adding markers of hemolysis (LDH) and absolute reticulocyte count (ARC). The hematological response was evaluated at six, 12 and the last six months of eculizumab treatment for patients with enough follow-up and a complete record (n=127).
Our data demonstrated that only 21% of patients had reached a complete or major response (no transfusion, normal hemoglobin values); about 67% fell into the two intermediate categories

(good and partial response), with hemoglobin values between 10 and 12 g/dL, evidence of residual intravascular hemolysis, and occasional transfusions for the partial responders; while about 11% were classified as minor or non-responder, with a regular transfusion requirement.
It is important to stress that a hematological “non-response” does not necessarily mean the lack of clinical benefit, for example in the case of a reduction in the incidence of a thrombotic event. However, this analysis could be useful to understand the mechanisms underlying the “non-response” to better address therapeutic choices (e.g., change the administration regimen, use other inhibitors of the complement system).

2.1.2 Unmet clinical needs in PNH patients during eculizumab

We know that different factors may be contributing to residual anemia during treatment with eculizumab, such as chronic intravascular hemolysis, underlying bone marrow failure and C3-mediated extravascular hemolysis

2.1.2.1 Intravascular hemolysis in PNH patients during eculizumab

Although rare, some patients, mainly Japanese, have also been identified as carriers of a hereditary C5 polymorphism, making them resistant to eculizumab therapy [126].
In the approximately 10-15% of patients on stable treatment with eculizumab, residual anemia may result from chronic intravascular hemolysis occurring approximately one to two days before the next administration of eculizumab, with no evidence of trigger events of the complementary cascade activation. In these cases, the incomplete block of C5 is associated with a reduction in the plasma concentration of eculizumab, demonstrated 12-14 days after administration [127]. For this reason, this condition has been defined as the pharmacokinetic breakthrough [128,129]. Possible therapeutic strategies, from which the patient can benefit, include increasing the dose to 1200 mg or reducing dose intervals to 10-12 days [121,127,130].
Another condition, which can also contribute to residual and, sometimes more unpredictable, anemia is the phenomenon of pharmacodynamic breakthrough [128,129, 131]. The minimal chronic activation of the complement system, due to C3 tick-over, can present "exacerbations" in conjunction with infectious and/or inflammatory events, such as to produce a massive activation of the complement system, which exceeds the ability of eculizumab to block its activity, despite adequate plasma levels. In this condition, it can only be useful to treat the causes that trigger the process.

2.1.2.2 C3-mediated extravascular hemolysis

In all probability, the main cause of residual anemia in PNH patients on eculizumab treatment is C3-mediated extravascular hemolysis[122,132]. Central to this mechanism is the alternative pathway of complement activation. In this pathway, C3 protein spontaneously hydrolyses and leads to the formation of C3 convertase (this process is also known as tick-over of C3), which is not impacted by eculizumab, acting on C5. This determines the accumulation of C3 fragments on PNH RBCs, such as C3b through glycophorin A, for example. Consequently, PNH RBCs are covered by fragments of C3, which as opsonins are recognized by the reticulo-endothelial system of the spleen and liver, and phagocyted. This condition has been demonstrated in vivo with a reduction in the half-life of Chromium 51- labeled erythrocytes and, at the same time, showing an increased uptake of the radio compound at the hepato-splenic level [122,132,133]. This mechanism, which affects the hematologic response to Eculizumab in about 25-50% of patients, however, extremely different between subjects. It has been hypothesized that this variability may depend on inherited polymorphisms involving complement regulatory genes that could predispose to an increased individual susceptibility to extra-vascular C3 hemolysis. Rondelli et al [134] demonstrated that patients carrying a variant of CR1 gene (complement receptor 1 gene) have an inadequate response to Eculizumab therapy. Given the high number of proteins involved in the activation and regulation of the complement system (e.g., factor H, factor B, factor I, etc.), it is possible that, in the future, other gene variants associated with a better or worse response to Eculizumab therapy will be identified.

In the past, corticosteroids have been used, without success and with numerous side effects; splenectomy has certainly proved to be useful but with concerns about thrombotic and infectious risk, so it is not considered a routine therapeutic choice [135-137].

The C3-mediated extravascular hemolysis is currently an important field of investigation for new therapeutic strategies. In May 2021, after the results of the phase III trial (PEGASUS), see below, Food and Drug Administration (FDA) has approved the first C3-inihibitor, pegcetacoplan, developed by Apellis Pharmaceuticals, for the treatment of adults with PNH who are anemic after therapy with a C5 inhibitor for at least three months [138-140].

2.2.2.3 Bone marrow failure syndromes and PNH

Another cause of residual anemia is inadequate compensatory erythropoiesis, due to an underlying bone marrow failure syndrome, with a PNH clone associated.

AA and PNH can be considered two sides of the same coin; about 30-40% of PNH patients may present AA during their disease course [81]. However, the presence of a PNH clone in the context

of severe AA (SAA) does not change the therapeutic approach of SAA: the first option for patients younger than 40 years with a matched related donor should be bone marrow transplantation, whereas, for patients older than 40 years or lacking a matched related donor, immunosuppression (horse anti-thymocyte globulin and cyclosporine) remains the first-line treatment [107,109]. The onset of non-hemolytic anemia, neutropenia, and/or thrombocytopenia should require bone marrow aspiration and a cytological examination in order to find out not only AA, but also a clonal evolution to myelodysplastic syndrome or acute myeloid leukemia. Although rare, the risk of clonal evolution is approximately 1-5% [81].

3. NOVEL THERAPEUTIC STRATEGIES

Eculizumab has revolutionized the natural history of PNH, however, as already explained, there are still unmet needs. The great interest in new possible therapeutic strategies has led to the development of numerous pre-clinical and clinical trials, aiming to identify new molecules.

The objective of the new field of investigation is to improve the hematological response and quality of life for PNH patients.

The new molecules, currently being tested, have been classified according to the target of the complement system on which they act; we have novel terminal inhibitors, which target C5, and proximal inhibitors, which interfere with C3 or even further upstream (factor B and D of the alternative pathway).

Among the new agents anti-C5, which are currently being tested for PNH, there are monoclonal antibodies, small peptides and small interfering RNA (siRNA).

These products aim to reproduce the excellent results achieved with eculizumab, trying, in the same way, to improve the compliance of patients. These drugs, in fact, are designed to increase dose intervals and to simplify the route of administration (e.g., oral or subcutaneous).

On the other side, we have novel proximal complement inhibitors, such as peptides and small molecules, designed to interfere with the early phase of complement activation, aimed at preventing chronic extravascular hemolysis in particular.

3.1 New terminal complement inhibitors

ALXN1210 (Ravulizumab-Alexion Pharmaceuticals)

ALXN1210 is a next-generation inhibitor of complement cascade that targets C5. Compared with eculizumab, it has an amino acid substitution able to modify its pharmacokinetics [141]. Due to this change in its aminoacidic sequence, the ALXN1210 molecule is more resistant to

degradation by lysosomes, when internalized by the cell, thus increasing its half-life. Two phase Ib/II studies were conducted to evaluate the efficacy and safety of ALXN1210, administered intravenously in naive PNH patients.

In the first study (NCT02598583, study 103), the drug was administered to 13 PNH patients every four weeks at a dose of either 900 mg (such as eculizumab) or 1,800 mg; in the second study (NCT026605993, study 201), 26 patients were treated with a maintenance dose of 1,000 mg every four weeks, 1,600 mg every six weeks, 2,400 mg every eight weeks, or 5,400 mg every 12 weeks. A rapid reduction in LDH values (which was the primary endpoint) was obtained in all treatment cohorts, especially in the cohort at a dose of 1,000 mg every four weeks. The safety profile is comparable to eculizumab, no adverse event was recorded.

Based on these preliminary data, Alexion designed two phase III randomized trials (study 301, NTC02946463 and study 302, NCT03056040). The first, a non-inferiority study of ALXN1210 compared with eculizumab, directed to naive PNH patients with signs of hemolysis (defined as LDH values >1.5 times the upper limit of normal (ULN)), enrolled 246 subjects, randomized 1:1 to receive either ravulizumab (at the dose of 2,700 +/- 300 mg in case of major changes in body weight) or eculizumab for six months. The primary composite endpoint was transfusion independence and normalization of LDH values. Ravulizumab was non-inferior to eculizumab for both endpoints (transfusion independence 73.6% vs. 66.1%; LDH normalization 53.6% vs. 79.3%, with the probability (p) for non-inferiority <0.001). It has also been shown to have excellent safety and tolerability with the administration schedule every eight weeks.

The second, study 302, designed as a switch-study, was directed to PNH patients already being treated with a standard dose of eculizumab, in stable clinical conditions, to evaluate the non-inferiority of ravulizumab. A total of 191 subjects were enrolled, randomized 1:1, who could switch to ravulizumab or remain on eculizumab treatment; the primary endpoint evaluated the reduction in LDH values. After six months of treatment, ravulizumab also confirmed non-inferiority in this patient setting, as well as further confirming the safety and tolerability profile. In light of these results, ravulizumab has received authorization from the Food and Drugs Administration (FDA) and European Medicines Agency (EMA)[142-148].

SKY59/RO711268/ Crovalimab (Hoffmann-La Roche)

Crovalimab is a recycling humanized anti-C5 monoclonal antibody designed for extended self-administered subcutaneous dosing of small volumes in diseases amenable for C5 inhibition, even in Japanese patients carrying the gene variant (p.Arg855His), which makes them resistant to eculizumab. This molecule is being tested in a phase I/II study (NCT03157635), divided into

three sequential parts, and an open-label extension, to assess the safety, pharmacokinetics, pharmacodynamics, and exploratory efficacy in healthy volunteers (part 1), as well as in complement blockade-naive (part 2) and C5 inhibitor-treated patients (part 3).

The first data regarding the study have recently been published, showing that SKY59 is an effective C5 inhibitor, with excellent bio-availability when administered subcutaneously (both weekly and at longer intervals); hemoglobin values increased by 1g/dL in untreated patients and remained stable in patients who were already being treated with eculizumab. A few episodes of breakthrough with intravascular hemolysis have been observed, especially in conjunction with trigger events of the complement system activation. No major safety issues were observed, except possible self-limiting vasculitis-like symptoms, associated with drug-target-drug immune complexes observed in patients switching from eculizumab to crovalimab [149-154].

Based on these results, La Roche designed three phase III trials: the first is a multicenter, single arm study aimed to assess the efficacy, safety, pharmacokinetics and pharmacodynamics of crovalimab in PNH naive patients (NCT04654468- COMMODORE 3); the second is a randomized, open-label, active-controlled, multicenter study designed to evaluate the non-inferiority of crovalimab compared with eculizumab in PNH patients currently treated with complement inhibitors (NCT04432584- COMMODORE 1); the third is a randomized, open-label, active-controlled, multicenter study designed to evaluate the non-inferiority of crovalimab, compared with eculizumab, in participants with PNH who have not been previously treated with complement inhibitor therapy (NCT04434092- COMMODORE 2) [155-157].

Coversin (Akari)

Coversin is a small protein complement C5 inhibitor, which prevents the cleavage of C5 by C5 convertase into C5a and C5b, but binds C5 at a different site, also active in patients carrying the C5 polymorphism. On the encouraging results of a phase I study, in healthy volunteers, coversin was tested in a phase II trial, in PNH patients with resistance to eculizumab due to complement C5 polymorphisms (NCT02591862-COBALT). Patients were treated with coversin by daily subcutaneous injection for six months to determine the safety and efficacy of the drug. Coversin was well tolerated, with rare reactions at the injection site, no antibodies neutralizing the molecule were found. LDH values decreased in all patients, although only two (out of 29) reached the primary endpoint of the study (LDH <1.8 ULN).

No patient required transfusion support, although a share of intravascular hemolysis was present. Coversin certainly has biological efficacy in PNH, a good safety profile and the possibility of being self-administered. Based on these results, Akari Therapeutics designed a phase III randomized open label trial (NCT03588026-CAPSTONE) to evaluate the safety and efficacy in transfusion-dependent PNH patients. The study was completed at the end of 2020, but the results are not yet available [158-163].

Table 1. Inhibitors of C5 in development in PNH

Compound	Structure and ROA	Clinical ID	Design	Schedule	Patient population *	Primary endpoint
ALXN1210 Ravulizumab	mAb, IV	NCT02598583 (study 103)	Phase Ib-II, open-label	DE	Naïve patients	Safety, efficacy (by LDH)
		NCT02605993 (study 201)	Phase Ib/II, open-label	MAD	Naïve patients	
		NCT02946463 (study 301)	Phase III random VS Ecu	Every 8 W	Naïve patients	Efficacy (by LDH)
		NCT03056040 (study 302)	Phase III random VS Ecu	Every 8 W	Stable PNH responders	
SKY59/ RO7112689 Crovalimab	mAb, IV and SC	NCT03157635 (COMPOSER)	Phase I- II multi-part study	SAD	Part 1 Healthy volunteers	Safety, tolerability PK, PD
				DE by IV infusion, followed by SC injection	Part 2 Naïve patients Part 3 Stable responders	
		NCT04432584 (COMMODORE 1)	Phase III random VS Ecu	IV loading-dose, followed by SC injection	Poor responders (by LDH <2x ULN)	Efficacy (by LDH)
		NCT04434092 (COMMODORE 2)	Phase III random VS Ecu		Naïve patients	
		NCT04654468 (COMMODORE 3)	Phase III		Naïve patients	
rVA576 Coversin	Small protein, SC	NCT02591862 (COBALT)	Phase II open-label	Daily	Poor responders (by LDH <1.5x ULN)	Efficacy (by LDH)
		NCT03588026 (CAPSTONE)	Phase III	Daily	Poor responders (by LDH <1.5x ULN and transfusion dependent)	Efficacy (by Hb)

* Stable or poor response is reported to standard eculizumab treatment. Abbreviations: ROA, route of administration; Ecu, eculizumab; Ab, monoclonal antibody; SC, subcutaneous; IV, intravenous; DE, dose escalation; MAD, multiple ascending dose; MD, multiple doses; SAD, single ascending dose; LDH, lactate dehydrogenase, ULN, upper limit of normal; W, weeks BID, bis in die (twice a day), TID, ter in die (thrice a day).

3.2 New proximal complement inhibitors

APL-2/ Pegcetacoplan (Apellis)

APL-2 is a pegylated version of the first-generation compstatin POT-4, with possible long-lasting action. On the results of a phase I study, in healthy volunteers, two studies were conducted in PNH patients: PHAROAH (NCT02264639), in subjects treated with eculizumab with inadequate response (defined by hemoglobin (Hb) <10g/dL and transfusion requirements); and PADDOCK (NCT02588833), dedicated to naive PNH patients with significant hemolysis (defined by LDH values >2x ULN).

From the published results, LDH values returned to normal in 95% of patients by the 28th day of treatment, maintaining this result throughout the study. Hemoglobin values also improved by about 2g/dL from the baseline; only a few patients required transfusion support. The control on both intra- and extravascular hemolysis with normalization of bilirubin values, reduction of reticulocyte count and increase in the PNH population is evident [164-168].

Based on these data, Apellis designed a phase III study PEGASUS (NCT03500549) for PNH patients with inadequate response to eculizumab therapy (defined for Hb values <10.5mg/dL), with the possibility, after a period of combined therapy, to be randomized to either eculizumab or APL-2, alone. Pegcetacoplan was superior to eculizumab with respect to the change in hemoglobin level from the baseline to week 16, with an adjusted (least squares) mean difference of 3.84 g per deciliter ($p<0.001$). A total of 35 patients (85%) receiving pegcetacoplan, compared with six patients (15%) receiving eculizumab, no longer required transfusions. Non-inferiority of pegcetacoplan to eculizumab was shown for the change in absolute reticulocyte count, but not for the change in lactate dehydrogenase level [138,139].

ACH-4471/ACH-044471/ALXN2040 (Achillion/Alexion)

ACH-4471 is a small oral factor D inhibitor developed by Achillion, which has shown in vitro hemolysis blocking capacity and a good pharmacokinetic profile. On the results of a phase I study, in healthy volunteers, treated with single and multiple ascending doses, 10 naive PNH patients with signs of intravascular hemolysis were enrolled in the phase II study (NCT03053102). PNH patients received danicopan monotherapy (100-200 mg three times daily). The purpose of this study was to determine the safety and efficacy of ACH-4471 through LDH and hemoglobin values. Intravascular hemolysis was inhibited, demonstrated by mean decreased LDH (5.7 times ULN at baseline vs. 1.8 times ULN [day 28] and 2.2 times ULN [day

84]; both p<0.001). Mean baseline hemoglobin, 9.8 g/dL, increased by 1.1 (day 28) and 1.7 (day 84) g/dL (both p<0.005) [169-173].

This phase II monotherapy data shows proximal inhibition with danicopan provides clinically meaningful intravascular hemolysis inhibition and hemoglobin improvement in untreated PNH patients, without evidence of C3-mediated extravascular hemolysis.

In parallel, there is a phase II study (NCT03472885) in PNH patients with inadequate response to treatment with eculizumab (assessed with transfusion needs), who were administered three different doses of the drug (100-150-200 mg three times a day). The primary endpoint of the study was to evaluate the increase in hemoglobin values. The addition of danicopan led to clinically and statistically significant reductions in the frequency of RBC transfusions and in the number of transfusion units in patients, compared with a history of eculizumab treatment alone [174,175].

A Phase II trial is also ongoing (NCT04170023) with second-generation Factor D (FD) inhibitor analogue (ALXN2050/ACH-0145228), in monotherapy in patients with PNH that are treatment naive, or patients currently treated with eculizumab who still experience anemia and reticulocytosis, or patients currently treated with ALXN2040 (danicopan) as monotherapy.

A phase III trial is now ongoing (NCT04469465).

This is a randomized, double-blind, placebo-controlled, multiple-dose study in PNH patients who have clinically evident extravascular hemolysis on a C5 inhibitor (eculizumab or ravulizumab).

The main objective of this study is to evaluate the efficacy of danicopan as an add-on therapy to a complement component 5 (C5) inhibitor.

Participants will be randomized to receive danicopan or a placebo, in a 2:1 ratio for 12 weeks, in addition to their C5 inhibitor therapy. At Week 12, patients randomized to receive the placebo will be switched to danicopan, in addition to their C5 inhibitor, for an additional 12 weeks. Participants randomized to danicopan will continue on danicopan for an additional 12 weeks, while remaining on their ongoing C5 inhibitor therapy.

At Week 24, participants may enter a one-year long-term extension period and continue to receive danicopan, in addition to their C5 inhibitor therapy [176,177].

Table 2. Anti-C3 and proximal inhibitors in development in PNH

Compound	Target	Structure and ROA	Clinical ID	Design	Schedule	Patient population *	Primary endpoint
APL-2 Pegcetacoplan	C3	Pegylated peptide, SC	NCT02264639 (PHAROAH)	Phase Ib, open-label	MAD, daily	Poor responders (by Hb <10 gr/dl and transfusion requirement)	Safety and tolerability
			NCT02588833 (PADDOCK)	Phase Ib, open -label	MAD, daily	Naïve patients	
			NCT03500549 (PEGASUS)	Phase III random VS Ecu	Twice weekly or every three days	Poor responders (by Hb <10,5 gr/dl)	Efficacy (by Hb)
LNP023 Iptacopan	FB	Small molecule, oral	NCT03439839	Phase II, open-label	BID	Poor responders (by LDH <1.5x ULN)	Efficacy (by LDH)
			NCT04820530 (APPOINT)	Phase III	BID	Naïve patients	Efficacy (by Hb)
			NCT04558918 (APPLY)	Phase III random VS Ecu	BID	Poor responders (by Hb <10, gr/dl)	
ACH-4471/ ACH-04471/ ALXN2040 Danicopan	FD	Small molecule, oral	NCT03053102	Phase II open-label	TID	Naïve patients	Efficacy (by LDH)
			NCT03472885	Phase II	MD, TID	Poor responders (by transfusion requirement)	Efficacy (by Hb)
			NCT04170023 (second-generation FD)	Phase II	TID	Part 1 Naïve patients Part 2 Poor responders Part 3 Patients on ACH471-103	
			NCT04469465	Phase III random Ecu	MD, TID	Poor responders (by Hb <9,5 gr/dl and transfusion requirement)	

* Stable or poor response is reported to standard eculizumab treatment. Abbreviations: ROA, route of administration; Ecu, eculizumab; Ab, monoclonal antibody; SC, subcutaneous; IV, intravenous; DE, dose escalation; MAD, multiple ascending dose; MD, multiple doses; SAD, single ascending dose; LDH, lactate dehydrogenase, ULN, upper limit of normal; W, weeks BID, bis in die (twice a day), TID, ter in die (thrice a day).

B. CLINICAL DEVELOPMENT OF IPTACOPAN

1. INTRODUCTION

Hemolysis in PNH is mainly due to complement-dependent intravascular hemolysis, which normally is blocked by CD59 preventing the final stage of complement assembly. Without the GPI anchor, CD59 is less expressed on the cell surface hence allowing for MAC formation and erythrocyte lysis [178]. In untreated PNH patients, the anemia is dominated by the intravascular hemolysis [179]. In patients treated with eculizumab, formation of the terminal complex is blocked, but there is accumulation of erythrocytes opsonized with C3 fragments, which makes them prone to undergo extravascular hemolysis [122.] In particular, C3dg fragments are strong signals for erythrophagocytosis [180].

Indeed, patients with PNH treated with C5-blockade (eculizumab) often develop a Coombs-positive hemolytic anemia that is C3-positive, IgG-negative [122]. The ongoing hemolysis is a burden for the patients leading to severe anemia, iron deficiency, pain due to vasospasm and fatigue.

Treatment of PNH with eculizumab, drastically reduce the intravascular hemolysis, but the patients show evidence of being sub optimally treated for their anemia. Thus, serum LDH levels remain elevated in most patients, 50% of patients are anemic and some require intermittent transfusions [127.] PNH patients with extravascular hemolysis often display erythrocytes opsonized with C3, C3b, iC3b and C3dg, the direct Coombs-test is often positive, there is a high percentage of reticulocytes, bilirubin is elevated and the LDH levels are slightly elevated [122] . LNP023 blocks the alternative pathway and prevents C3 fragments from opsonizing the erythrocytes. Thereby, patients with PNH should experience less of extravascular and intravascular hemolysis [179, 181].

1.1 Iptacopan: the molecule and preclinical studies

LNP023 (Iptacopan-Novartis) is a novel oral small molecular weight compound that inhibits Factor B of the alternative complement pathway. In vitro, it inhibits the lysis and opsonization of the red cells mediated by C3[182]. The first in human Phase I trial (CLNP023X2101) was designed to assess safety, tolerability, pharmacokinetics (PK) and pharmacodynamics (PD) of LNP023 after single and multiple oral dose administration to healthy volunteers (HV). LNP023 in HV appeared safe and was well tolerated.

1.2 Iptacopan: the clinical development

The molecule was studied, in a phase II trial (NCT03439839), in PNH patients showing signs of active hemolysis (defined for LDH values > 1.5 ULN) despite treatment with eculizumab. The study aimed to evaluate the safety, efficacy, pharmacokinetic and pharmacodynamic profile of the molecule. LNP023 is administered at a dose of 200 mg twice a day. The primary end-point was to evaluate the effect of Iptacopan on the reduction of chronic residual intravascular hemolysis, estimated through the LDH values after 13 weeks of treatment. At 13 weeks, patients could opt for a long-term study extension (ongoing), allowing for modification of standard treatment (eculizumab)[183-185].

1.3 Iptacopan: initial data on combination treatment

In this multicentric, open label, single arm study, 10 patients were enrolled. Iptacopan was well tolerated, without any major drug-related safety findings, and showed LDH reduction (mean 539 international units per liter (IU/L) [standard deviation (SD) 263] vs. 235 IU/L [44], change from baseline -309.2 IU/L [SD 265.5], 90% confidence interval (CI) -473.77 to -144.68, p=0.0081) and hemoglobin normalization (mean 97.7 g/L [SD 10.5] vs. 129.5 g/L [18.3] change from baseline 31.9 g/L [14.5], 90% CI 23.42-40.28, p<0.0001) in most PNH patients at week 13 [183].

On the basis of these data, we have two phase III trials ongoing. The first (NCT04558918) is a randomized, multicenter, active-comparator controlled, open label trial, aimed at determining whether LNP023 is efficacious and safe for the treatment of PNH through the demonstration of superiority of LNP023, compared with an anti-C5 antibody treatment in adult PNH patients presenting with residual anemia, despite treatment with anti-C5 therapy. The second (NCT04820530) is a single arm, open label study aimed at determining whether LNP023 is efficacious and safe for the treatment of PNH-naive patients [185,186].

Blockade of the AP with oral LNP023 has the potential to prevent both intra – and extravascular hemolysis and may, therefore, offer therapeutic alternative to the existing eculizumab therapy that currently require intravenous infusions every two week.

2. IPTACOPAN: OUR INVESTIGATION

Here we have conducted a systematic long-term analysis of the phase II study, aiming to investigate the long-term use of iptacopan, and mostly systematically evaluate its efficacy and

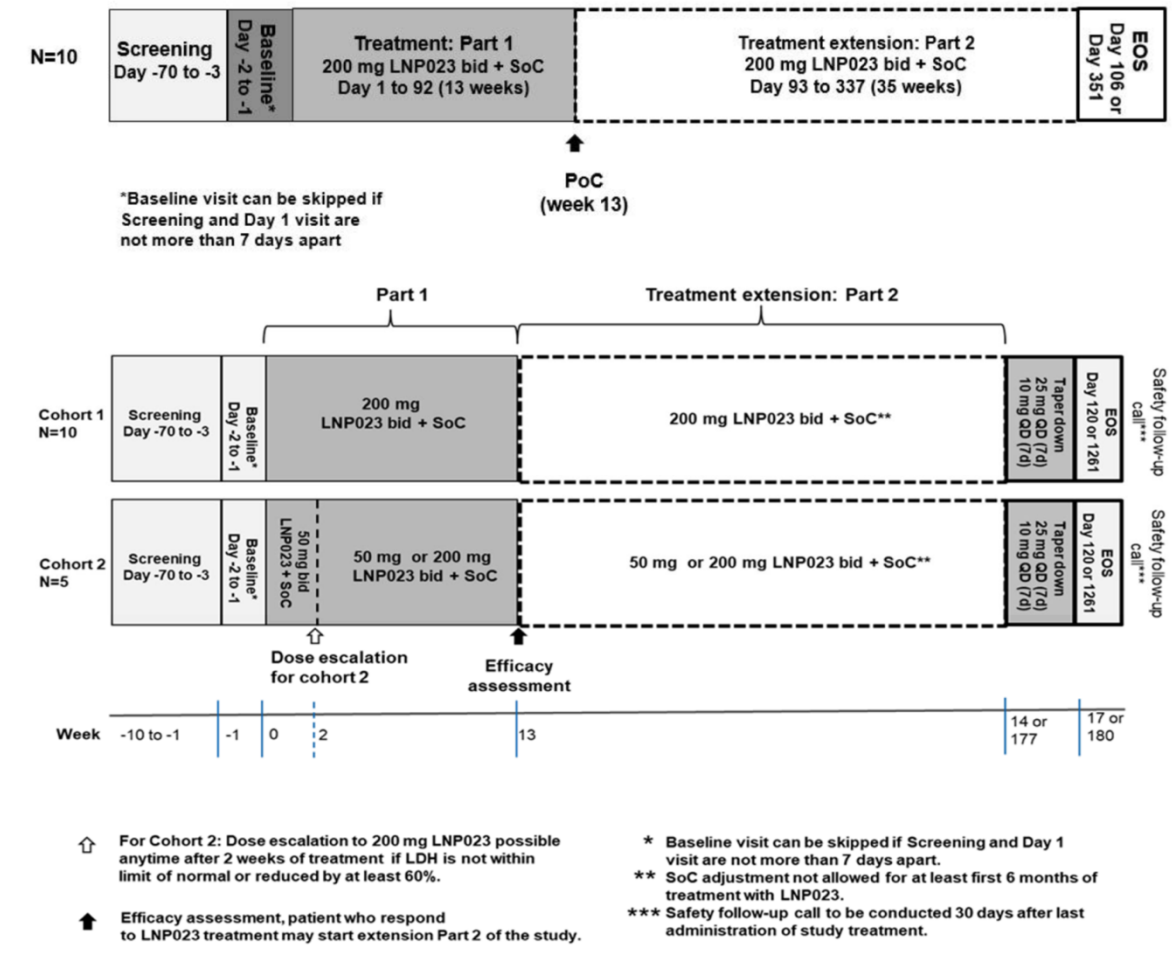
safety in monotherapy. This is an exploratory analysis conducted on a limited cohort of patients representing 40% of total enrollment, including all the patients enrolled at our site. For this purpose, we combined clinical and biological objectives, setting the following specific aims:

1. To evaluate the efficacy in monotherapy of LNP023 in reducing and controlling chronic intra- and extra-vascular hemolysis in PNH patients, showing signs of active hemolysis despite treatment with eculizumab.

2. To analyze C3- deposition on RBCs and changes in PNH clone size, in patients treated with LNP023 in monotherapy.

3. MATERIAL AND METHODS

3.1 Study design



The study began in 2018 and is, to date, still ongoing in its extension phase. As explained above, it is a phase II, multicenter, multiple-dose, proof-of-concept study. The first part of the study, which began on May 31, 2018, included a treatment cohort of 10 PNH patients, enrolled at

Federico II University Hospital (Naples, Italy), Hôpital Saint-Louis (Paris, France), and University Hospital Essen (Essen, Germany), with active hemolysis, assessed according to LDH values >1.5 ULN, on stable treatment with eculizumab, treated with LNP023 at 200 mg twice a day, The main objective of the study was to evaluate, at 13 weeks of treatment, the efficacy of the novel complement system factor B inhibitor, iptacopan, in reducing chronic intravascular hemolysis.

The 13-week results were published in Lancet Haematology in May 2021 (see above) [183].

In light of the interesting and encouraging results obtained in the first 13 weeks, the 10 enrolled patients were proposed to continue the experimental treatment in an extension phase, which is ongoing to date, with the option to discontinue treatment with eculizumab and to continue only LNP023.

In addition, it was decided to enroll a second treatment cohort (10 patients), starting in June 2019, to whom LNP023 was administered at a lower dosage of 50 mg twice a day, with the possibility of increasing the dosage to 200 mg twice a day in case of failure to normalize blood parameters. All patients increased the dosage to 200 mg twice a day to achieve a deeper inhibition of hemolysis. As with the cohort 1 patients, those in cohort 2 were allowed to continue the experimental treatment in an extension phase after and to modify the therapy with eculizumab.

3.2 Population

3.1.2 Inclusion criteria

Patients with the following criteria were considered eligible for the study:

1. Male and female patients between the age of 18-80 (inclusive) with a diagnosis of PNH based on documented clone size of ≥10% by RBCs and/or granulocytes, measured by GPI-deficiency on flow cytometry.

2. PNH patients on stable regimen of Eculizumab for at least 3 months prior to first treatment with LNP023.

3. For Cohort 1 only: LDH values ≥ 1.5x upper limit of the normal range for at least 3 pre-standard of care (eculizumab) dosing measurements taken in relation to 3 different SoC dosing dates over a maximum of 10 weeks prior to Day 1. All other screening pre-SoC LDH values have to be >1x upper limit of normal range.

4. For Cohort 2 only: LDH values ≥ 1.25x upper limit of the normal range for at least 3 pre-SoC dosing measurements taken in relation to 3 different SoC dosing dates over a maximum

of 10 weeks prior to Day 1. All other screening pre-SoC LDH values have to be >1x upper limit of normal range.

5. For Cohort 2 only: Hemoglobin level <10.5 g/dL at baseline.

6. Previous vaccination against Neisseria meningitidis types A, C, Y and W-135, against N. meningitidis type B, against H. Infuenzae and S. Pneumoniae. If LNP023 treatment has to start earlier than 4 weeks post vaccination, prophylactic antibiotic treatment must be initiated.

7. For Part 2 of the study, patients who as per judgment of Investigator benefit from LNP023 treatment based on reduced hemolytic parameters as compared to Screening and Baseline.

3.2.2 Exclusion criteria

Patients with the following characteristics were excluded from enrollment:

1. Patients with laboratory evidence of bone marrow failure (reticulocytes <60x10E9/l, platelets <30x10E9/l neutrophils <1x10E9/l).
2. Known or suspected hereditary complement deficiency at screening
3. History of hematopoietic stem cell transplantation
4. A positive HIV, Hepatitis B (HBV) or Hepatitis C (HCV) test
5. Severe concurrent co-morbidities, e.g. patients with severe kidney disease (dialysis), advanced cardiac disease (NYHA class IV), severe pulmonary arterial hypertension (WHO class IV), unstable thrombotic event not amenable to active treatment as judged by the investigator.

3.3 Endpoints

As endpoints, we analyzed:
1. LDH values at the last available follow-up of monotherapy treatment;
2. Hb level and transfusion requirements at the last available follow-up of monotherapy treatment;
3. Other laboratory parameters, indicators of residual hemolysis, such as reticulocyte count, haptoglobin, total bilirubin;
4. C3 deposition on RBCs;
5. The PNH clone size .

As exploratory endpoint, we assessed the hematologic response achieved by patients on LNP023 treatment, estimated according to the new categories recently proposed by the EBMT SAAWP [124].

3.4 Follow-up procedures

The study included weekly follow-up visits for the first six weeks, then every two weeks for the first six months, then once a month.

At each visit the patients underwent physical examination, routine blood tests (complete blood count with reticulocyte count, LDH, haptoglobin, total and direct bilirubin), collection of biological samples (peripheral blood) to measure the size of the PNH clone and C3 deposition on erythrocytes, and detailed collection of information regarding the signs and symptoms related to the disease. All biological samples were collected before taking tablets of LNP023.

3.5 Statistical analysis

Long-term data are presented descriptively, individually, by calculating mean and SD of the cohort; the only comparison was made with student paired T test (for paired samples) comparing for each parameter the data at the last administration of eculizumab (combination therapy) with the data collected at the last available follow-up (monotherapy).

4. RESULTS

At our center eight patients were considered eligible, initially enrolled at the AOU Federico II in Naples and then moved to the AORN Moscati in Avellino, where they are still under treatment. They received the experimental treatment LNP023/*iptacopan* (at different dosage depending on the treatment arm) in addition to eculizumab (at a dose of 900 or 1200 mg ev, every two weeks).

Patients were in two different treatment arms: 4 patients (cohort 1) were receiving LNP023 at a dose of 200 mg twice a day in addition to eculizumab; the other 4 (cohort 2) were receiving LNP023 at a dose of 50 mg twice a day in addition to eculizumab. All patients in cohort 2 used the option of increasing the dose to 200 mg twice a day to achieve a deeper inhibition of hemolysis.

All patients (cohorts 1 and 2) accepted to continue experimental treatment in the extension period, all at the dose of 200 mg twice a day.

Three of four patients in cohort 1 discontinued eculizumab therapy after 12 months of combination therapy and continued therapy with iptacopan. The one ,who did not discontinued eculizumab, because was concomitantly diagnosed with a squamous cell carcinoma of the tongue, on a preexisting lesion that was present before the iptacopan start date, and in the

interest of patient continued the investigational treatment in combination therapy, until he died for cancer progression.

The four patients in cohort 2 discontinued eculizumab therapy after 6 months of combination treatment.

To mitigate the possible risk of infectious complications, all patients were vaccinated for Neisseria meningitidis, Haemophilus influenzae, and Streptococcus pneumoniae and could receive antibiotic prophylaxis.

Our patient cohort is composed of 8 patients (6 male/ 2 female), (age 26-65 years) treated with LNP023, first in combination with eculizumab and then in monotherapy, at a dosage of 200 mg twice daily.

At baseline, patients had the following characteristics (tables 3 and 4): mean values of hemoglobin (8,75 g/dl [DS 1,22]), of LDH (1143,5 U/L [DS 1346,0]) (equal to 4,7 times ULN), of reticulocytes (256,87 10*9/L [DS 103,2]), and total bilirubin (3,34 mg/dl [DS 1,66]).

All patients had an undetectable haptoglobin (<0.075 g/L).

Six out of eight patients had received an erythrocyte transfusion in the 12 months before enrolment (mean 6 packed erythrocyte units per year, range 0-20). Three patients were receiving eculizumab at dose of 1200 mg every two weeks, while 5 at dose of 900 mg every two weeks.

Patients had a mean PNH clone value on RBCs of 56.83%, [SD 24,18], on granulocytes of 94,34%, [SD 8,52] and a mean PNH RBCs C3+ value of 24,86 [SD 17,19]. The PNH RBCs and PNH granulocytes ratio had a mean value of 0,58.

According to the recent response categories proposed by Risitano et al. [124] to assess the efficacy of eculizumab, at baseline four out of eight patients were considered no responders, one out of eight was a minor responder, and three out of eight were considered partial responders.

Table 3. Baseline demographics and characteristics

Parameter	001	003	004	005	006	007	008	009
Age, years	42	26	35	52	65	38	62	48
Sex	M	M	M	M	M	F	F	M
Time since diagnosis, years	19	11	12	29	17	1	4	10
N° RBC transfusions in the year prior to start LNP023	8	2	3	20	9	6	0	0

Hb pre-treat g/dl	10,4	9,3	9,6	8	6,4	8,9	9,3	8,1
LDH pre-treat U/L	661	2873	3698	373	398	340	262	543
Total bilirubin pre-treat mg/dl	2,6	4	3,5	1,38	3,37	1,85	6,7	2,3
Reticulocyte count pre-treat $x10^9$/L	151	398	278	196	130	220	403	279
Haptoglobin pre-treat g/L	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08
PNH granulocytes , % FLAER	99%	98%	98%	99,9%	91%	75%	99,5%	98,8%
PNH monocytes, % FLAER	99%	96%	97%	99,8%	94%	85%	99,5%	98,8%
PNH erythrocytes, CD59	51,74%	66,48%	80,66%	61,46%	38,41%	23,19%	95,98%	37%
PNH RBC C3+ %	33,19%	18,96%	42,01%	19,50%	7,51%	2,88%	53,9%	21%
PNH RBC/PNH granulocytes ratio	0,52	0,67	0,82	0,61	0,42	0,30	0,96	0,37
Eculizumab treatment at baseline	900 mg	1200 mg	1200 mg	900 mg	1200 mg	900 mg	900 mg	900 mg

Hemoglobin reference range 12-16 g/dL; LDH reference range 125-243 U/L; Total bilirubin reference range 0.6-1.1 mg/dL; Reticulocyte count reference range 50-120 × 109/L; Haptoglobin reference rang 0,07- 2,34 g/L

Table 4. Baseline charactertics

Population: n = 8 Caucasian patients	
Age, years	
Mean (DS)	46 (13,4)
Median (range)	45 (26-65)
Sex	
Male	6 (75%)
Female	2 (25%)
Hb, g/dl	
Mean (DS)	8,75 (1,22)
Median (range)	9,1 (6,4-10,4)
LDH U/L	
Mean (DS)	1143,5 (1346,0)
Median (range)	470 (262-3698)
Reticulocyte count $x10^9$/L	
Mean (DS)	256,87 (103,2)
Median (range)	249 (130-403)
Total bilirubin mg/dl	
Mean (DS)	3,34 (1,66)
Median (range)	3,34 (1,38-6,7)

Number of RBC transfusions in the year prior to start LNP023	
Mean (DS)	6 (6,61)
Median (range)	4,5 (0-20)
PNH granulocytes , % FLAER	
Mean (DS)	94,34 (8,52)
Median (range)	98,4 (75-99,9)
PNH erythrocytes, CD 59	
Mean (DS)	56,83 (24,18)
Median (range)	56,6 (23,12-95,8)
PNH RBC/PNH granulocytes ratio	
Mean (DS)	0,58 (0,22)
Median (range)	0,56 (0,30-0,96)
PNH RBC C3+ %	
Mean (DS)	24,86 (17,19)
Median (range)	20,25 (2,88-42,01)

Results at last follow-up of combination therapy

At the last follow-up before discontinuation of eculizumab (at 12 months from the start of combination therapy for patients in cohort 1 and at 6 months for patients in cohort 2), patients presented the following characteristics (tables 5 and 6): mean value of hemoglobin (13,1 g/dl [DS 0,68]) vs 8,75 g/dl [DS 1,22] at baseline, p=1,501; of LDH (271,75 U/L [DS 61,66]) ,equal to 1,1 times ULN, vs (1143,5 U/L [DS 1346,0]) at baseline, p= 0,106 ; and mean values of reticulocytes (137,12 10*9/L [DS 40,37]), and of total bilirubin (1,54 mg/dl [DS 0,92]).

No patients required transfusion therapy. Patients had a mean PNH clone value on RBCs of 91,8%, [DS 9,09] vs 56.83%, [SD 24,18] at baseline, p=0,001; mean values on granulocytes 96%, [DS 9,4] and a mean PNH RBCs C3+ value of 0,30 [DS 0,39] vs 24,86 [SD 17,19] at baseline, p= 0,005. The PNH RBCs and PNH granulocytes ratio had a mean value of 0,94.

Trying to estimate the hematological response, at the last follow-up of combined treatment, of the 8 patients, 7 achieved a complete response, 1 achieved, instead, a major response.

Table 5. Results at last follow-up of combination therapy

Parameter	001	003	004	005	006	007	008	009
Treatment duration (months)	12	12	12	12	6	6	6	6

Hb g/dl	14,2	13,7	12,8	12,6	12,8	13,3	12	13,4
LDH U/L	282	379	230	165	296	258	261	303
Total bilirubin mg/dl	2,16	1,02	0,52	1,15	3,37	1,80	1,6	0,7
Reticulocyte count pre-treat $x10^9$/L	231	121	139	144	130	108	104	120
Haptoglobin g/L	0,38	0,08	0,08	0,12	0,08	0,08	0,08	0,08
PNH granulocytes , % FLAER	99,9%	98%	99%	99,8%	93%	86%	99,5%	99%
PNH monocytes, % FLAER	99,9%	96%	99%	99,7%	94%	84%	99,5%	99%
PNH erythrocytes, CD59	99,9%	96,7%	95,3%	98,1%	80,1%	76,0%	98,6%	89,7%
PNH RBC C3+ %	0,06%	0,08%	0,18%	0,41%	1,22%	0,33%	0,09%	0,09%
PNH RBC/PNH granulocytes ratio	1	0,98	0,96	0,98	0,86	0,88	0,99	0,89

Hemoglobin reference range 12-16 g/dL; LDH reference range 125-243 U/L; Total bilirubin reference range 0.6-1.1 mg/dL; Reticulocyte count reference range 50-120 × 109/L; Haptoglobin reference rang 0,07- 2,34 g/L.

Table 6. Results at last follow-up of combination therapy

Population: n = 8 Caucasian patients	**Last follow-up ecu + LNP023**
Hb, g/dl	
Mean (DS)	13,1 (0,68)
Median (range)	13,05 (12-13,7)
LDH U/L	
Mean (DS)	271,75 (61,66)
Median (range)	271,5 (165-379)
Reticulocyte count $x10^9$/L	
Mean (DS)	137,12 (40,37)
Median (range)	125 (104-231)
Total bilirubin mg/dl	
Mean (DS)	1,54 (0,92)
Median (range)	1,37 (0,52-3,37)
PNH erythrocytes,	
Mean (DS)	91,8 (9,09)
Median (range)	96,0 (76-99,9)
PNH RBC C3+ %	
Mean (DS)	0,30 (0,39)
Median (range)	0,13 (0,06-1,22)
PNH granulocytes , % FLAER	
Mean (DS)	96 (4,9)
Median (range)	99 (86-99,9)
PNH RBC/PNH granulocytes ratio	
Mean (DS)	0,94 (0,05)
Median (range)	0,97 (0,86-1)

Results at last monotherapy follow-up

The analysis concerns the seven patients who switched to iptacopan in monotherapy, since one patient did not discontinue eculizumab since he was not considered clinically stable given the concomitant cancer. As described below, no significant differences were observed for all parameters evaluated during combination therapy end the results obtained during monotherapy treatment (available for 7 patients), after the discontinuation of eculizumab. In particular, these 7 patients maintained hemoglobin values almost in the normal range with no evidence of clinical hemolysis, remaining transfusion free.

At last follow-up (December 2021- January 2022), patients presented the following characteristics (tables 7, 8 and 9): mean values of hemoglobin (13,83 g/dl [DS 1,35]) vs (13,17 g/dl [DS 0,71]) at last follow-up of combination therapy, p=0,1159 , mean values of LDH (304 U/L [DS 65,59]) vs (287,75 U/L [DS 47,59]), p=0,4788; mean values of reticulocytes (121,42 10*9/L [DS 37,16]) vs 136,12 [DS 43,51], p= 0,3575; and mean values of total bilirubin (1,2 mg/dl [DS 0,60]) vs 1,59 (DS 0,98), p= 0,2645.

No patients required transfusion therapy. Patients had a mean PNH clone value on RBCs of 98,65%, [DS 1,38] vs 90,75%, [DS 9,30], at last follow-up of combination therapy, p=0,07; on granulocytes 9,3%, [DS 0,69] and a mean PNH RBCs C3+ value of 0,12% [DS 0,39] vs 0,29 % [DS 0,41] at last follow-up of combination therapy, p=0,1781.

Trying to estimate the hematological response again, at the last follow-up, of the 7 patients, 6 achieved a complete response, 1 achieved, instead, a major response.

Table 7. Results at last monotherapy follow-up

Parameter	001	003	004	006	007	008	009
Treatment duration (months)	42	42	42	30	30	24	24
Hb g/dl	14,9	13,5	13,7	14,5	14,4	11	14,8
LDH U/L	385	350	290	354	279	188	285
Total bilirubin mg/dl	2,5	0,9	0,8	1,4	1,0	1,6	0,8
Reticulocyte count pre-treat $x10^9$/L	180	150	110	140	100	104	70
Haptoglobin g/L	0,08	0,08	0,08	0,08	0,08	0,08	0,08
PNH granulocytes , % FLAER	98,9	98,2	99,1	99,9%	99,7	100%	99,9%
PNH monocytes, % FLAER	98,9	98,6	99,1	99,9%	99,9	100%	99,9%
PNH erythrocytes, CD59	99,8	96,4	98,1	99,1%	99,9	100%	97,5%

PNH RBC C3+ %	0	0,04	0	0,41	0,33	0,01	0,09
PNH RBC/PNH granulocytes ratio	1	0,98	0,98	1	1	1	0,97

Hemoglobin reference range 12-16 g/dL; LDH reference range 125-243 U/L; Total bilirubin reference range 0.6-1.1 mg/dL; Reticulocyte count reference range 50-120 × 109/L; Haptoglobin reference rang 0,07- 2,34 g/L

Table 8. Results at last monotherapy follow-up

Population: n = 7 Caucasian patients	**Last follow up LNP023 monotherapy**
Hb, g/dl	
Mean (DS)	13,83 (1,35)
Median (range)	14,4 (11-14,9)
LDH U/L	
Mean (DS)	304 (65,59)
Median (range)	290 (188-385)
Reticulocyte count x10^9/L	
Mean (DS)	121,42 (37,16)
Median (range)	110 (70-180)
Total bilirubin mg/dl	
Mean (DS)	1,2 (0,60)
Median (range)	1,0 (0,80-2,50)
PNH erythrocytes %	
Mean (DS)	98,65 (1,38)
Median (range)	98,6 (96,4-100)
PNH RBC C3+ %	
Mean (DS)	0,12 (0,39)
Median (range)	0,17 (0-0,41)
PNH granulocytes , % FLAER	
Mean (DS)	99,38 (0,69)
Median (range)	99,7 (98,2-100)
PNH RBC/PNH granulocytes ratio	
Mean (DS)	0,99 (0,01)
Median (range)	1 (0,97-1)

Table 9.

Table 9 Population: 7 patients*	At last dose of eculizumab	At last monotherapy follow-up	P value (T student test)
LDH U/L			
Mean (DS)	287 (47,59)	304,42 (65,59)	0,4788
Median (range)	283 (230-379)	290 (188-385)	

Hb g/dl Mean (DS) Median (range)	 13,17 (0,71) 13,3 (12-13,7)	 13,83 (1,35) 14,4 (11-14,9)	 0,1159
Total bilirubin mg/dl Mean (DS) Median (range)	 1,59 (0,98) 1,6 (0,52-3,37)	 1,2 (0,60) 1,0 (0,80-2,50)	 0,2645
Reticulocyte count x109/L Mean (DS) Median (range)	 136,12 (43,51) 121 (104-231)	 121,42 (37,16) 110 (70-180)	 0,3575
PNH erythrocytes, CD59 Mean (DS) Median (range)	 90,75 (9,30) 94,98 (76,02-99,9)	 98,65 (1,38) 98,6 (96,4-100)	 0,0729
PNH RBC C3+ % Mean (DS) Median (range)	 0,29 (0,41) 0,09 (0,06-1,22)	 0,12 (0,39) 0,17 (0-0,41)	 0,1781

Hemoglobin reference range 12-16 g/dL; LDH reference range 125-243 U/L; Total bilirubin reference range 0.6-1.1 mg/dL; Reticulocyte count reference range 50-120 × 109/L.

* Data are only on 7 patients whose eculizumab therapy was discontinued.

Safety

In our study three serious adverse events were observed, two of which were in the same patient. No serious adverse events were considered to be related to iptacopan. One patient developed an acute renal injury after screening but before starting iptacopan; the event was associated with a massive hemolytic crisis, which was probably triggered by the vaccines administered before the iptacopan start date as per the study protocol. The event resolved without sequelae, and the patient started the iptacopan treatment. During the study treatment, the same patient had an intracranial bleeding, which was probably secondary to transient warfarin overdosing (given because of past history of multiple thromboembolisms) associated with thrombocytopenia. This event was clinically severe, presenting with headache followed by seizures and systemic inflammation associated with complement activation as shown by laboratory signs of haemolysis . Iptacopan treatment was not discontinued and no clinical signs or symptoms of hemolysis have been observed, showing a fully compensated subclinical breakthrough episode. All laboratory parameters eventually normalized within 5 weeks once the adverse event resolved without sequelae. Another patient received the diagnosis of squamous cell carcinoma of the tongue, on a preexisting lesion that was present before the iptacopan start date. The patient underwent complete surgical excision but had local relapse within 7 months and he unfortunately died a few months later . We reported a few mild infectious episodes, treated with

appropriate therapy and resolved without sequelae. We reported no thrombotic event, with the caveat of limited follow up.

5. DISCUSSION AND CONCLUSIONS

Eculizumab, the first anti-C5 monoclonal antibody approved for patients with paroxysmal nocturnal hemoglobinuria (PNH), has revolutionized the natural history of this disease, blocking intravascular hemolysis, reducing the risk of thrombo-embolic events, resulting in a significant improvement in survival and quality of life. However, the hematological response to eculizumab is extremely heterogeneous, with only one-third of PNH patients reaching normal hemoglobin levels.

It is important to stress that a hematological “non-response” does not necessarily mean the lack of clinical benefit, for example in the case of a reduction in the incidence of a thrombotic events [123].

However, we know that different factors may be contributing to residual anemia during treatment with eculizumab, such as chronic intravascular hemolysis, C3-mediated extravascular hemolysis and underlying bone marrow failure.

The field of novel anticomplement inhibitors is developing rapidly and has great potential to improve treatment and to address the above questions.

In PNH patients on eculizumab treatment, the combined therapy with LNP023 has been shown to be effective in terms of hematological response, leading to the normalization of hemoglobin values, reducing intravascular hemolysis, demonstrated by the almost normal LDH levels, and also acting on extravascular hemolysis, assessed in vitro by the disappearance of RBCs C3-covered. The blocking of all mechanisms of hemolysis is also demonstrated by the increase in clone size, which in some patients is greater than 95%. This resulted in improvement of anemia without transfusion requirements. The same results were confirmed during the extension period in monotherapy. The proportion of PNH RBCs seen on iptacopan has never been observed with any other treatment, and it is the proof of the highest biological efficacy (all RBCs escape from complement mediated lysis, either intravascular or extravascular). Look for the ratio PNH RBC/PNH gran, which is close to 1 (compare with baseline).

In addition, administration in two daily doses, appears to be "convenient" for patients.

The results of our study are extremely encouraging about control on both intra- and extra-vascular hemolysis, but the main concern is the safety profile. At the moment, no major issues have been reported; anti-infectious strategies, including a broader vaccination schedule, as well as possible pharmacological anti-microbial prophylaxis, are probably useful. However, a longer follow-up is required to rule out the possible impact on the risk of infectious complications, auto-immune diseases, as well as cancer.

Another interesting aspect in PNH patients on proximal complement inhibitors is the increased survival of PNH red blood cells, due to a control on both intra- and extra-vascular hemolysis. While this condition results in increased levels of hemoglobin – and therefore in a better hematological response and, in all probability, an improved clinical condition for the patient – we do not yet know the risk of breakthrough hemolysis in this condition, especially in monotherapy. Current data seem to be extremely encouraging about the control of hemolysis, even with a large PNH erythrocyte population, but breakthrough episodes may develop both in the case of subtherapeutic drug level (just think, banally, in case of a missed dose), or in particular conditions, such as during infectious episodes [187]. Breakthrough hemolysis has not been observed, with the caveat of limited follow up; minimal residual intravascular hemolysis, as seen by LDH, remains negligible, since mild LDH increase is expected considering the huge PNH RBCs mass susceptible to lysis.

New drugs are showing strong impact on unmet clinical needs, the new challenge is to tailor therapy according to disease and patient specific features. Further studies are needed to understand the “new scenarios” of PNH, especially during novel complement inhibitors, so as to choose the best approach for our patients.

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