DEVELOPMENT OF THE FIRST-IN-CLASS ORAL FACTOR D INHIBITOR DANICOPAN FOR THE TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

Dottoranda: **Docente Tutor:**

Dott.ssa Luana Marano   **Ch.mo Prof. Antonio Maria Risitano**

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1.1. Paroxysmal Nocturnal Hemoglobinuria (PNH)

1.1.1 Definition and historical notes.

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare acquired hematological disorder of the hematopoietic stem cells, characterized by chronic complement-mediated intravascular hemolysis, thromboembolic events affecting mostly venous vessels and bone marrow failure of different degrees [1-4].

One of the first descriptions of the disease was made by Dr. Paul Strübing, who described in 1882 a 29-year-old man presenting with fatigue, abdominal pain and severe episodes of hemoglobinuria. At that time, Dr Strübing already suggested a possible mechanism of intravascular hemolysis [5] that in the following years was discovered to be the basis of the disease. In 1911, the Italian physicians 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 in 1925 that Enneking introduced the term "Paroxysmal nocturnal hemoglobinuria" (PNH) [8]. In 1937, Thomas Ham observed that erythrocytes from PNH patients, when incubated with normal acidified serum, hemolyzed [9]. This fundamental discovery led to the creation of the first diagnostic test, which still bears his name (Ham Test). Doctor Ham hypothesized that the lysis of cells incubated with acidified serum was complement-mediated since heat leads to the inactivation of the reaction. However, he could prove it only when the alternative pathway of the complement system was identified, in 1954 (by Pillemer and others) [10]; until then only the antibody-mediated activation pathway was known, while Ham's studies showed that erythrocyte hemolysis in acidic serum of patients with PNH was an antibody-independent process. The 1980s brought 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 normally expressed on normal erythrocytes, but missing PNH erythrocytes. In particular, two proteins were identified: CD55 and CD59, as regulators of the complement system, characterized by their expression on the cell membrane through the glycosyl phosphatidyl-inositol (GPI) anchor. A few years later, the genetic mutation (phosphatidylinositol glycan class A [PIG-A]) responsible for the deficiency

of the GPI anchor proteins was identified, as this gene encodes an enzyme that serves in a fundamental step in the synthesis of GPI anchor, which therefore represents the missing molecular element on the erythrocytes of these patients [11-14].

1.1.2 Epidemiology

PNH is considered a rare disease, with the estimated incidence of 1-5 cases per million individuals per year; however, PNH could have been underestimated in the past due to diagnostic difficulties. Over the years, the diagnostic methods (from the Ham test to flow cytometry) and the awareness of clinicians in considering PNH differential diagnosis of different clinical condition (even in the lack of the typical hemoglobinuria) have improved. This helped to increase the identification of PNH blood cell populations (often referred as "PNH clones"), an aspect that in the near future may lead to a more reliable evaluation of the incidence of the disease [15,16].

There are no particular differences in its prevalence between the various geographical areas; a slight increase in the number of cases is reported in some Asian regions, for example Thailand, areas where there is also an increased incidence of aplastic anemia. PNH can affect any age although it is frequently observed in young adults, with no apparent difference between male and female gender (although the PIG-A gene is located on the X chromosome, the probability of developing the disease is the same, as the mutation occurs in somatic cells in which the active chromosome X is always only one), and there is no hereditary transmission precisely because the mutation does not pertain the germ cells [17,18].

1.1.3 Etiology and molecular basis

Paroxysmal nocturnal hemoglobinuria 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 from Kinoshita, and then described in detail in 1994 by Bessel Bessler [14,19-22].

The PIG-A gene encodes an essential enzyme that promotes the transfer of N-acetyl-glucosamine onto phosphatidyl-inositol, necessary for the formation of glycosyl-phosphatidyl-inositol (GPI), a glycopeptide used by the cell as an anchor system for numerous proteins physiologically located on the outer layer of the plasma membrane. As a consequence of the mutation, the affected stem cells and their progeny cell are totally or partially deficient of the proteins anchored to the membrane by GPI ("GPI-anchored proteins", GPI-APs) [23,24].

Furthermore, the PIG-A gene is the only one located on the X chromosome, so that it is possible to produce a cell totally devoid of the enzyme with a single mutation acquired both in males and females (in males due to the presence of only one chromosome X, in females due to the inactivation of the second X). Generally, each patient carries a single mutation, although multiple clones with different mutations can rarely coexist. Most PIG-A mutations are small insertions or deletions, usually 1 or 2 bp, resulting in frameshift mutations from the mutated site onwards, with the consequent synthesis of a functionless product, thus leading to cells totally lacking GPI. However, missense mutations may also be present, a fact that leads to the production of a minimal amount of functional GPI product [25].

Cells totally devoid of GPI are named PNH type III cells, while those still capable of producing small amount of GPI are named type II; type I, on the other hand, defines cells with a normal GPI-APs expression profile. Mutations of type II cells are almost necessarily missense mutations with single amino acid substitution, while large deletions or nonsense mutations usually result in the generation of type III cells. Obviously, this difference in expression will also lead to a total deficiency of proteins anchored with GPI in type III, but an only partial one in type II, a characteristic that determines for type II erythrocytes also a lower susceptibility to complement-mediated lysis. It has been observed that it is possible that clones with type II and III mutations are present in the same patient 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 [26,27].

Small GPI negative clonal populations have also been documented in healthy individuals; similarly, such populations are quite frequent in patients with immune-mediated aplastic anemia (about 30-40% of patient may harbor a PNH population). From this observation it can be inferred that the presence of the genetic alteration alone is not sufficient to determine the pathology. Clonal expansion starting from a single hematopoietic cell would seem to be due to the fact that the mutated cells are capable of resisting to some insult, probably of an immune nature, to which the normal cell is sensitive (figure 1).
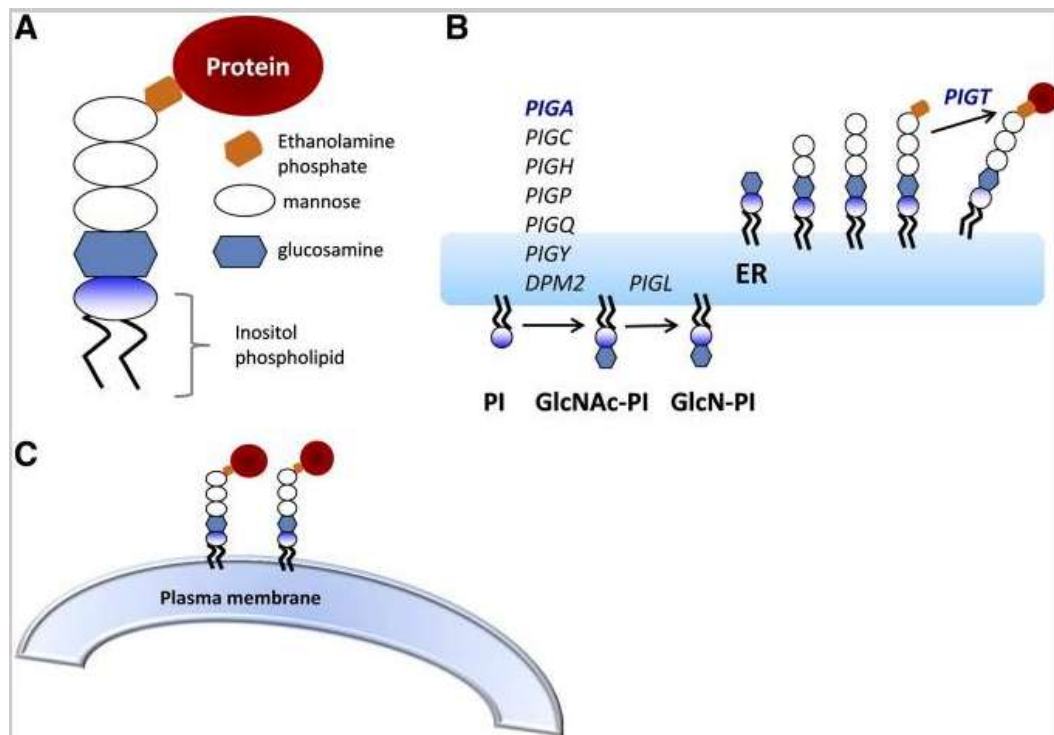
Figure 1. Synthesis of the GPI anchor: In all PNH cells the synthesis of the glycosylphosphatidinositol anchor is impaired, therefore all PNH cells do not express on their surface the proteins bound through the GPI anchor. (Brodsky, Blood 2014)

1.1.4 PNH phenotype and "the escape theory"

The mutation of the PIG-A gene is therefore the characteristic of the disease, but this mutation, although necessary, is not sufficient to cause the 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 [28,29];

- ✓ 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 gradually disappear after the interruption of the treatment [30];

- ✓ The expansion of the PNH population is difficult to reproduce in mouse models; a knock-out animal cannot be generated, since 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 [31-34].

✓ PNH hematopoiesis is certainly clonal but not necessarily monoclonal.

In fact, in some cases it 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 presenting the same functional defect, but which are molecularly heterogeneous, could be compatible either with a random process or with a selection process of the PNH phenotype stem cell versus the normal stem cell [35-38].

All these evidences have led to the formulation of the hypothesis of the double pathophysiology of PNH, also known as "relative advantage" or "escape theory" [3,4,39].

According to this theory, a mutation of the PIG-A gene may be a relatively common phenomenon 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 [40]. External factors can alter this balance, creating a permissive environment for the expansion of the PNH stem cell, which can, therefore, support hematopoiesis even for the patient's entire life [41]. 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 the acquired bone marrow failure syndromes the "normal" hematopoietic stem cells are subject to an attack, probably of an immunological nature, the target of which has not yet been identified, nor has the mechanism that determines the loss of tolerance been understood; this leads to an exhaustion of the hematopoietic compartment and consequent cytopenia. Unknown triggers lead to a cellular immune response, which results in an expansion of clones of T lymphocytes, 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 [42-44].

It is reasonable to assume, then, that a stem cell can occasionally and randomly acquire a mutation of the PIG-A gene, but this does not translate into the expression of the disease due to the lack of intrinsic proliferative advantage of the PNH stem compared to the normal one, unless a second event occurs, in this case an immunological attack, 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 guarantees normal

hematopoiesis. As a consequence of the depletion of the physiological compartment, the PNH stem cell, preserved from immunological attack, can expand. Therefore, the expansion of the PNH stem cell is proposed as an "escape" mechanism against the immunological attack aimed to the hematopoietic stem cell and the exclusive consequence of the negative selective pressure exerted on normal stem cells. Indeed, one may also 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).
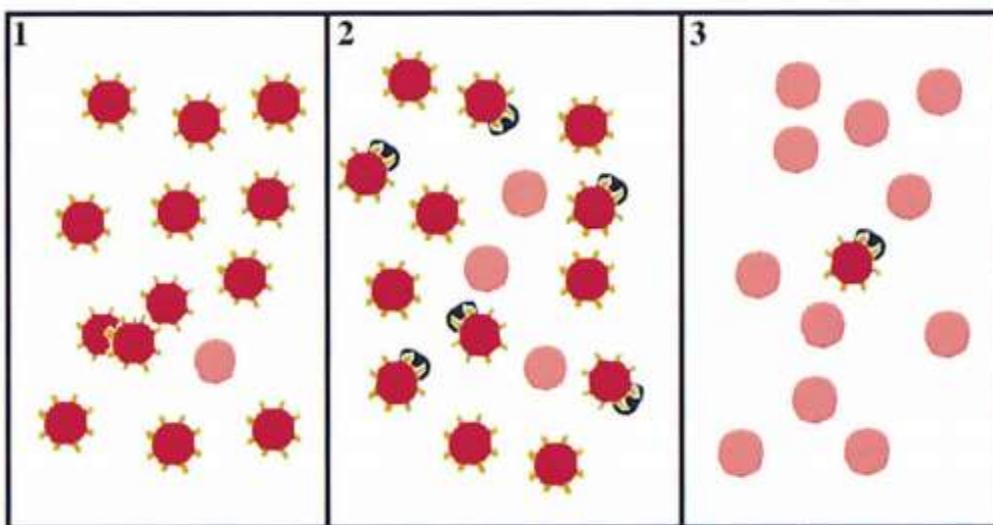
Figure 2. Theory of double physiopathology (Rotoli, Luzzatto 1989)

In this image, normal hematopoietic cells (HSCs) are shown in dark red, showing protrusions on their surface, 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. The black symbols represent "hypothetical" molecules which, by binding the GPI-APs, lead to the destruction of the HSCs.

Panel 1: a normal marrow is shown, in which some mutated PIG-A cells may coexist.

Panel 2: Normal HSCs suffer an attack that targets GPI-APs. The mutated HCs-PIG-A are not affected by the attack. Hematopoiesis becomes a mosaic of normal cells and PNH.

Panel 3: The persistence of negative selective pressure on normal HSCs leads to the relative expansion of mutated HCs-PIG-A; thus, hematopoiesis becomes predominantly PNH phenotype.

1.1.5 PNH and Aplastic Anemia (AA)

Bone marrow failure is the other clinical feature of PNH; indeed, almost half of PNH patients also exhibit AA at some time during the disease course. On the other hand, up to 40% of AA patients may harbor a PNH cell population, which may appear at any stage during the disease course, and may possibly lead to clinical PNH [45]. The classification of PNH proposed by the

International PNH Interest Group [46] includes a spectrum of disease categories according to the presence of clinical bone marrow failure and hemolysis (figure 3).
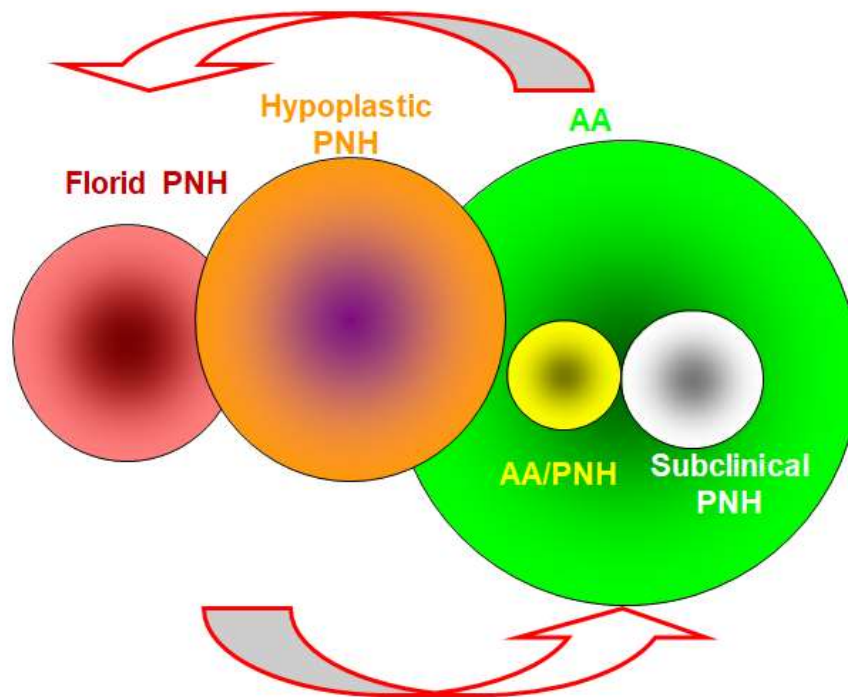
Figure 3. Clinical overlap between aplastic anemia and PNH (A.M. Risitano)

These categories range from classic PNH (purely hemolytic, no clinical bone marrow failure) to subclinical PNH in the context of AA (minor PNH population without meaningful hemolysis), with AA/PNH syndrome [47] and a large intermediate group with varying degrees of marrow failure and hemolysis [48]. To note, this close link between AA and PNH is more than a simple association, given that common pathogenic mechanisms are involved in AA and PNH. According to the well-established 'dual pathophysiology theory' [3,21], an immune-mediated damage is considered needed to allow the relative expansion of the few PIG-A mutated HSCs. Indeed, this auto-immune attack targets normal hematopoiesis, but it would spare PNH HSCs, eventually resulting in their progressive expansion, which may limit the development of clinical bone marrow failure, but on the other hand, eventually leads to the clinical phenotype of PNH.

1.1.6 Pathogenesis

In the pathogenesis of paroxysmal nocturnal hemoglobinuria, two regulators of the complement system play a fundamental role among the GPI- anchored proteins: CD59/MIRL (Membrane Inhibitor of Reactive Lysis) and CD55/DAF (Decay accelerating Factor) [49-52].

CD55, a 68000 KDa glycoprotein, controls the complement cascade at an early stage, inhibiting the formation of C3 convertase, and promoting its dissociation once it is formed; CD59, a 19000 KDa glycoprotein, instead acts in the terminal phase of the cascade, blocking the incorporation of C9 into the C5b-C8 complex and thus preventing the formation of the membrane attachment complex (MAC) [53-56]. Due to the absence of CD55 and CD59, PNH red blood cells (RBCs) are more susceptible to complement-mediated lysis and this determines the chronic intravascular hemolysis that these cells undergo, with a consequent reduction in the half-life of affected RBCs. Notably, there is a constant minimal activity of the complement system caused by the so-called 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 capable of enormously amplifying the activity of the complement (such as inflammatory and / or infectious pathology) [57,58].

CD59 seems to play a predominant role, as evidenced by those very rare patients with hereditary defects of a single molecule: while the only patient described with a hereditary defect of CD59 had clinical manifestations similar to PNH, the CD55-deficient subjects on the surface of the red blood cells (a relatively common condition called the Inab phenotype) show no signs of hemolysis. This is also confirmed by data obtained in vitro: red cells with the Inab phenotype were incubated in acidified serum and the hemolysis and deposition of C3 were measured (as an activation index of the complementary cascade). Inab red blood cells, which express normal levels of CD59, do not undergo hemolysis in acidified serum, even if they show an accumulation of C3 on the surface 20 times higher than that seen in the testing. When CD59 is inhibited using a specific antibody, the accumulation of C3 drastically increases, and 100% of Inab red blood cells develop hemolysis in acidified serum. These data also confirm the primary role of CD59 in controlling hemolysis mediated by complementary activation [59-61].

It is still not entirely clear why the exacerbations of the hemolytic crises occur at night; the nocturnal crises that gave the pathology its name are typical. However, the lowering of the blood pH due to the retention of CO_2 already identified by Ham may be involved (the increased urinary concentration of the 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) as well as on the surface of red blood cells. However, there is no evidence of a

reduction in the half-life of PNH WBCs, probably due to the presence of additional molecules, possibly non-GPI-linked (CD46), or to other mechanisms that protect these cells from complement-mediated lysis [52,62]. Instead, the activation of the complement cascade on the platelet surface has been proposed as a possible cause of the tendency to develop thrombotic episodes, characteristic in patients with PNH. 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 hypothesis have been proposed to explain thrombophilia in PNH; one cause for example could be an alteration of the fibrinolytic system linked 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 the formation of plasmin. The development of thrombotic episodes could also be directly linked to intravascular hemolysis, as a consequence of the increase in plasma of free hemoglobin that binds and reduces NO concentrations; in this way, the anti-aggregating, anti-adhesive and vasodilating effect characteristic of NO would be lost. Another hypothesis sees, during the complement activation and consequent hemolysis, the release of microvesicles by RBCs, PLTs and WBCs and also by endothelial cells (even though the mechanism is still unclear), which in turn serve as pro-coagulating substances. 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 [25,58,63-68].

1.1.7 Clinical and natural history

The peripheral blood of PNH patients is a mosaic of affected and unaffected mature blood cells. The degree of mosaicism is determined by the level of expansion of the mutated clone(s), but the factors that determine the degree of clonal expansion in the individual patient are unknown. Although PNH is a clonal disorder, it is not a malignant disease and, for unknown reasons, the size of expansion of the mutant clone for PIG-A is highly variable in different patients. In some cases more than 90% of peripheral blood cells may derive from the clone, while in other cases less than 10% of circulating cells have a defect in the expression of GPI-APs. The variability in the extension 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 consistent clones often present important symptoms as a consequence of chronic massive intravascular complement-mediated hemolysis and require specific therapies with complement inhibitors.

From a clinical point of view, PNH is characterized by a typical triad: hemolytic anemia and related signs and symptoms, propensity to thromboembolic phenomena and bone marrow failure.

Based on the different clinical presentations found in various patients, the following classification of the disease, proposed by the International PNH Interest Group [46], is widely used today:

- ✓ Classical PNH, in which the hemolytic picture predominates and there is not bone marrow dysfunction;
- ✓ 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 (also here AA or myelodysplasia)

This classification, however, has limitations in use, as sometimes the clinical presentation can fall into more than one described category, or alternatively in none of them. 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 may have overt, variable hemolysis pictures typical of the classical form, or more nuanced pictures 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 expressed with different severity, based on the size of the PNH clones, on the sensitivity to complement-mediated lysis of PNH reds (Type II or Type III), and on the degree of activation of the complement itself (which is widely variable over time based on conditions or by individual characteristics of the patient). Hemolysis is typically chronic, due to the aforementioned minimal spontaneous activation of the complement (C3 tick-over), with paroxysms related to trigger events generally consisting in infections or other acute inflammatory events. Intravascular hemolysis leads to a series of symptoms and signs, of which the most remarkable is undoubtedly hemoglobinuria, which is a result of the passage of hemoglobin released by lysed erythrocytes in the urine, which causes its typical dark color. According to the urinary concentration, the color can vary from a dark yellow or dark red to a frank black (typical of the first urine of the morning). The color of urine is often described as marsala (by Dr. Marchiafava in his pioneering description of PNH) or Coca-Cola color.

Regardless of hemolysis, the resulting anemia can be of variable and multifactorial degrees. This, in fact, depends on the degree of complement activation, on the compensatory capacity of residual erythropoiesis (depending on the level of bone marrow failure, but also on any deficient conditions, for example secondary to chronic iron loss through hemosiderinuria). This means that some patients have normal hemoglobin levels or at least within the normal limits, while others present a severe anemic state that requires intensive transfusion support.

Typical signs of intravascular hemolysis are jaundice, hemoglobinuria, increased LDH values, increased indirect bilirubin, decreased haptoglobin concentration, presence of hemosiderin in the urinary sediment and variable reticulocytosis, consistent with 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 with consequent contraction of the vascular and gastrointestinal smooth muscles. Frequent symptoms, closely related to the degree of anemia, are asthenia, fatigue, lethargy, that all possibly leads to a significant reduction in the quality of life [67,69].

Another typical manifestation of PNH is thromboembolic events, which are the main cause of morbidity and mortality in this pathology. The propensity to venous thromboembolic phenomena is characterized by the involvement of atypical locations, 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 different in the population, with a reduced frequency in Southeast Asia compared to Europe and the United States. This could be related to the complex pathophysiology of such events, as described above, and to the possible contribution of various genetic predisposing factors [70].

Several observations have been reported, among the predisposing factors for thrombosis, massive intravascular hemolytic crises and PNH clones on large WBCs. However, even patients with a small population have an increased thrombotic risk compared to healthy individuals [69,71].

Finally, in several PNH patients cytopenia other than anemia may be present, whose severity depends on the degree of underlying bone marrow failure, which may be from moderate to the extent of the development of severe aplastic anemia, requiring specific treatment. The clinical manifestations of cytopenia depend on the affected line 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 a reduced functional capacity of the same [72].

The natural history of paroxysmal nocturnal hemoglobinuria is not predictable due to its heterogeneity in presentation and clinical course. Average survival time after diagnosis of an untreated individual is 10 years, with about 25% of patients reaching a survival time 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 (particularly cerebral thrombosis 25% and S. di Budd-Chiari 23%) and infectious complications (25%) [73-75].

1.1.8 Diagnosis

The diagnosis of PNH remains a clinical diagnosis and should be suspected in patients with moderate to severe anemia with reticulocytosis, elevated LDH levels, moderate jaundice, and negative Coombs test. The presence, moreover, of dark urine (Coca-Cola or marsala colored) (figure 4), of hemosiderin in the urinary sediment, of a variable picture of leuko-thrombocytopenia and, finally, of a positive anamnesis for thromboembolic phenomena affecting atypical venous sites is strongly suggestive of PNH. In particular conditions, PNH can even be suspected 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 shown, the first three frankly hemoglobinuric, Coca-Cola color, the others with different degrees of hemoglobinuria.

Diagnosis requires the documented partial or total absence of GPI-APs on the plasma membrane of at least two blood cell lines. The absence of the clone does not allow a diagnosis of PNH to be made; on the contrary, its presence must be evaluated in the patient's clinical context [76-77].

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 [78]. Although simple to perform, this test has several limitations; in fact, in addition to being technically difficult to standardize and requiring time and experience for its execution, it is not very sensitive, because it is difficult to highlight 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 [79].

Since early 1990s, the recognized method for identifying the PNH clone is flow cytometry, which through the use of monoclonal antibodies conjugated to fluorochromes, directed against the single GPI-APs, allows to effectively discriminate cells with total defect (type III) or partial (type II) 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 that show a deficit of expression of GPI-APs and to follow its trend over time. Finally, the sophisticated instruments available make it possible to achieve sensitivity such as to allow the identification of even small clones. The cell lines under study are neutrophil granulocytes, for the identification and correct quantification of the clone, the erythrocytes, mainly for the identification of the clone as the size can be underestimated due to hemolysis and/or transfusion phenomena, and monocytes, confirming the presence of the clone. Clone size is determined by the percentage of neutrophil granulocytes that do not express GPI-APs [16]. 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 separating them from normal platelets due to their small size [80-82]. For the assessment 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 carried out on peripheral blood anticoagulated with EDTA or heparin, preferably within 24-48 hours of 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 clone 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 picture.

1.2 Treatment of PNH

1.2.1 The pre-Eculizumab era

PNH treatment was mainly supportive until 2005, aimed at controlling the typical manifestations of the disease. The main problems to be managed are hemolysis and the consequent anemia, thrombotic phenomena and bone marrow failure. As regards of hemolysis and the consequent anemia, the therapeutic options were often unsatisfactory. Steroids have been widely used both in chronic and acute in conjunction with hemolytic crises [83,84]. Certainly steroids seem to give a benefit on symptoms related to hemolytic crises, such as dysphagia, abdominal pain, but nobody could demonstrate their role in reducing symptoms which often improve spontaneously as self-resolution of the paroxysmal crisis. Furthermore, there is no data showing that steroids do directly block the activation of the complement system and therefore hemolysis [46]. Their use, at a dosage of 0.5-1.0 mg/kg, is related to an important 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 of associated bone marrow failure [85,86]. However, the risk-benefit ratio must be adapted to the patient's clinical picture, taking into account side effects, such as liver toxicity, virilization, and, not to forget, the increased risk of developing Budd-Chiari syndrome [46]. Some patients have shown clinical benefit with the use of danazol given at a dosage of 400-600 mg/day.

Given 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 kept above 8 g/dl. This leads to an improvement in general symptoms, since higher hemoglobin levels reduce the stimulus to erythropoiesis with less production of PNH cells. Unlike polytransfused patients, in PNH patients, given the perpetual 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, as 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 [87]. Finally, the use of recombinant erythropoietin can help, if the endogenous levels are inadequate [84,88,89].

The treatment of thrombophilia represents one of the most debated points in the therapy of PNH, since 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 discussed is the treatment of the 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 plausible, given the unpredictability of thromboembolic events and the lack of studies to support these therapeutic strategies; the possible benefits must be balanced against the bleeding risk, given by warfarin therapy, in patients with low platelet concentrations. It is possibly reasonable to proceed with primary prophylaxis in those patients who have additional risk factors, (such as factor V of Leiden, familiarity, etc.). With regard to secondary prophylaxis, there is greater agreement in subjecting patients who have already had a thrombotic episode to anticoagulant therapy for life, although there are also different opinions on the best strategy. Many authors prefer to start from low molecular weight heparin and then move on to warfarin. Despite the different applicable strategies, as mentioned above, the recurrence rate of a thrombotic episode in patients with PNH is still very high [90]. In the treatment of an acute thrombotic episode anticoagulant drugs have been used at therapeutic doses similar to the treatment for other thrombosis [91], and in addition good results have been observed in some cases with fibrinolytic therapy.

In any case, as discussed below, the introduction of Eculizumab with the aim of treating complement-mediated hemolytic anemia has substantially changed also the management of thrombophilia of PNH.

With regard to the treatment of bone marrow aplasia, two supportive strategies with anti-infectious can be attempted, either anti-thrombotic and anti-hemorrhagic prophylaxis, or also etiological therapies based on different immunosuppressive regimens. In accordance with 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 attempted in patients with PNH: in some minor studies cyclosporine A showed some benefit, while other groups tried more intensive regimens using anti-thymocytic globulin combined with high-dose prednisone and cyclosporine A, showing mixed results for now.

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

Currently the only curative strategy for PNH patients is the transplantation of hematopoietic stem cells. It has been attempted since the 1980s and has proven effective in eradicating the PNH clone, being able to lead to recovery from the disease, while bringing 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 [92-94], large-scale prospective studies have been lacking and one of the biggest, in which 67 SCTs were performed in PNH patients, there are different donor types (syngenic, sibling or HLA-identical unrelated) and different conditioning regimens (myeloablative or reduced intensity), it showed a long-term survival of 75% [93].

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 [95]. In light of these results and the lack of studies on larger cohorts, it is difficult to identify guidelines for SCT in patients with PNH. At present, the main indication is the concomitant presence of bone marrow failure, and, as with AA, SCT should be considered first-line treatment when HLA-identical donor siblings are present, or in case of therapeutic failure in patients with an unrelated but HLA-matched donor [96-98]. Another factor to consider is the patient's age, given that the mortality and morbidity of the transplant increase along with the latter. 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; while the latter remains a good second-line choice for those who do not present an adequate response to the Eculizumab.

1.2.2 The "Eculizumab" era

Eculizumab (Soliris ®, Alexion Pharmaceuticals) is a humanized monoclonal antibody, which binds the C5 component of the complement system, preventing its cleavage into C5a and C5b and thereby blocking the formation of the membrane attack complex (MAC), responsible for the end-effector mechanism causing intravascular hemolysis of PNH erythrocytes [99] (figure 5). In addition, Eculizumab prevents the release of pro-inflammatory mediators, resulting from the cleavage of C5a. The blockade of 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 [100].
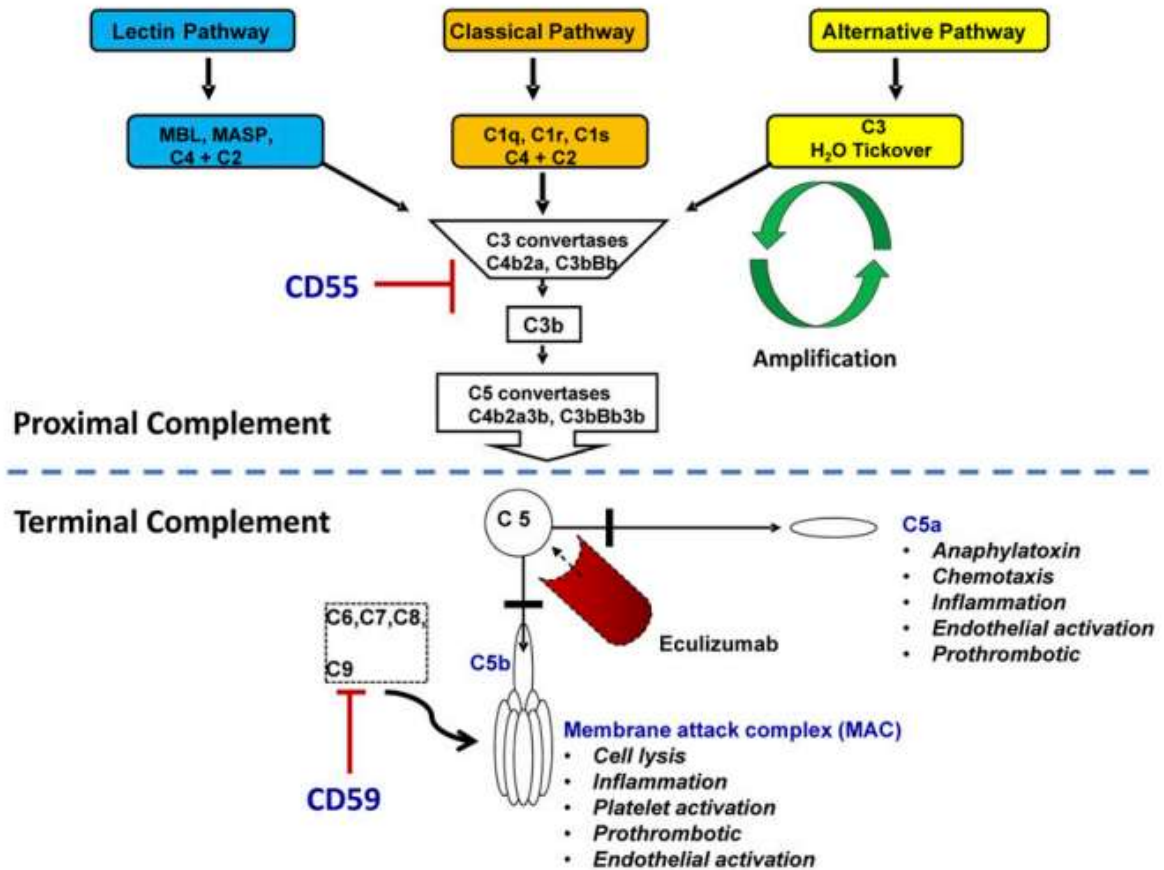
Figure 5. Complement cascade and mechanism of action of Eculizumab (Brodsky, Blood 2014)

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 red blood cells causes 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 4 weekly doses of 600mg, followed by a dose of 900mg every two weeks, starting from the fifth week.

The efficacy and safety of this drug have been amply 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 had a high transfusion requirement [101,102]. Subsequently, definitive evidence of efficacy was proven by two multicenter studies, TRIUMPH and SHEPERD.

The TRIUMPH, a double-blind, multicenter, randomized study enrolled 87 patients available to receive Eculizumab or placebo [103]. This study demonstrated the efficacy of Eculizumab in reducing intravascular hemolysis (measured by LDH levels), with an overall stabilization of hemoglobin levels at levels of independence from transfusions in about half of the treated patients, being also enormously effective in reducing general symptoms of the disease compared to placebo. These data were further confirmed first by the SHEPERD trial, an open-label study that included a larger population of PNH patients than the previous one, also including patients with minimal transfusion and thrombocytopenia requirements [104]. In this study were enrolled 97 patients, who received treatment for 52 weeks. Among the beneficial effects of the drug are certainly demonstrated: a reduction in hemolysis, with a reduction in average LDH 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. The overall transfusion requirement in the 2006 pivotal study decreased by 74%. These conditions lead to an appreciable improvement in anemia: about a third of patients reach hemoglobin levels close to normal, while another third remains free from transfusions, while continuing to have moderate anemia (this condition will be treated later). Another aspect evaluated was the incidence of thromboembolic phenomena following therapy with Eculizumab, a result relevant in all patients regardless of whether or not they were on anticoagulant therapy (respectively 94% vs 92% fewer thrombotic events). It is not known whether this reduction is attributable solely to an indirect action linked to the inhibition of hemolysis or to a specific effect with a real reduction in the patient's thrombophilic condition, and in this sense a significant reduction in plasma levels of coagulation activation markers has been reported, along with reactive fibrinolysis and endothelial cell activation in patients treated with the drug [105]. 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, historical cohort was compared with patients treated with Eculizumab, OS was 92% (95% CI, 87 to 98) at 6 years in the Eculizumab cohort versus 68%

(95% CI, 60 to 76) in the historical cohort [106] and rates of thrombosis reduced from 5.6 events to 0.8 events per 100 patient-years with Eculizumab treatment [107].

As for the safety and toxicity profile of Eculizumab, the drug appears to be free from significant side effects and to be well tolerated, however the risk of infectiousness remains high in patients under treatment, especially from encapsulated germs such as N. Meningitidis. This risk represents the main complication of the treatment and for this reason, meningococcal vaccination is recommended, using both the tetravalent conjugate vaccine specific for serotypes A, C, W and Y, and the vaccine for serotype B, before starting therapy, with references every three years [108].

Eculizumab is an expensive drug and must be administered throughout the patient's life in order to maintain a lasting response; therefore, patients with vague 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 initiate treatment [77].

1.2.3 Response categories

Response to Eculizumab can be extremely heterogeneous among patients, and different response categories can be identified. Already in 2009, Risitano et al [109], proposed a first classification of the hematological response in patients treated with Eculizumab: optimal response (no transfusion, hemoglobin> 11g/dL), good response (no transfusion, hemoglobin between 8 and 11 g/dL), partial response (transfusion requirement present, but reduced by at least 50% compared to baseline), and minor response (transfusion requirement unchanged or reduced by less than 50%). From this first categorization it emerged that only 1/3 of patients treated with Eculizumab reach normal hemoglobin values, a condition that led to investigate the reasons for this "limitation" of Eculizumab therapy. Approximately 10 years later, further data confirmed that Eculizumab treatment is effective and safe, but nevertheless leads to rather variable hematological responses [108,106,110]. Therefore, in addition to independence from transfusions, 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, the following new categories have been proposed [111]:

-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 8 and 10 g/dL, occasional transfusion support);

-minor response (regular transfusion requirement);

-no response (high transfusion requirement) (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)

1.2.4 Unmet clinical needs for PNH patients in the Eculizumab era

The treatment of PNH has certainly been revolutionized by the introduction of Eculizumab, however this antibody is not the cure for PNH and unmet clinical needs remain for many PNH patients, the response to Eculizumab can be extremely heterogeneous among patients. Although hemoglobin reaches stable values in most patients, also leading to transfusion independence, some patients, although showing an increase in hemoglobin values compared to baseline, remain meaningfully anemic.

It is important to emphasize that a hematological "non-response" does not necessarily mean the absence of clinical benefit; think of the drastic reduction in the incidence of thrombotic events in patients receiving Eculizumab. Understand the mechanisms underlying the "non-response" is the only possibility to better address therapeutic choices, different factors may be contributing to residual anemia during treatment with Eculizumab.

Although rare, some patients, mainly Japanese, have also been identified as carriers of a hereditary C5 polymorphism, making them resistant to Eculizumab therapy [112].

In the approximately 10-15% of patients on stable treatment with Eculizumab, residual anemia may result from chronic intravascular hemolysis occurring approximately 1-2 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 [113]. For this reason, this condition has been defined as the pharmacokinetic breakthrough phenomenon [114,115]. Possible therapeutic strategies, from which the patient can benefit, include increasing the dose to 1200 mg or reducing the administration intervals to 10-12 days [108,113,116].

Another condition, which can also contribute to residual and, sometimes more unpredictable, anemia is the phenomenon of pharmacodynamic breakthrough [114,115,117]. The minimal chronic activation of the complement system, due to the so-called C3 tick-over phenomenon, 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 act promptly on the causes that trigger the process.

Another cause of residual anemia is inadequate compensatory erythropoiesis, due to the presence of aplasia in bone marrow associated with the PNH clone, as already explained. Both a residual intravascular hemolysis due to a suboptimal block of C5, and an inadequate compensatory response of erythropoiesis may contribute to the residual anemia in PNH patients treated with Eculizumab, however, most have good control of intravascular hemolysis (evaluated with LDH values <1.5 times ULN) and similarly presents an adequate reticulocytosis (widely> 100,000/microL).

Considering the unresolved issues, the great interest in new possible therapeutic strategies has led to the development of numerous preclinical and clinical studies, aimed at identifying new molecules.

1.2.5 New inhibitors of the terminal portion of the complementary cascade

There are at least seven new anti-C5 agents (in addition to the Eculizumab biosimilar) that are currently being tested for PNH; many of these are monoclonal antibodies, small peptides and small interfering RNA (siRNA). All these agents aim to reproduce the excellent results achieved with Eculizumab, trying, in the same way, to resolve some issues especially regarding patients compliance; it has to be considered the need for intravenous

administrations every 14 days. These drugs, in fact, have been studied to increase the intervals of administration and to modify the route of intake (oral or subcutaneous).

ALXN1210 (Ravulizumab-Alexion Pharmaceuticals)

ALXN1210 is the first inhibitor of the second generation complement system that acts at the level of C5 and which, compared with Eculizumab, has an amino acid substitution which modifies its pharmacokinetics [118]. This change in the amino acid sequence makes the ALXN1210 molecule 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 4 weeks at a dose of either 900 mg (such as Eculizumab) or 1800 mg; in the second study (NCT026605993, study 201), 26 patients were treated with a maintenance dose of 1000 mg every 4 weeks, 1600 mg every 6 weeks, 2400 mg every 8 weeks, or 5400 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 1800 mg every 4 weeks. The safety profile is comparable to Eculizumab, no adverse event was recorded, except for an episode of meningitis resolved with appropriate antibiotic therapy, which however did not lead to the interruption of the experimental therapy. These preliminary results led to the design of two phase III randomized studies (study 301, NTC02946463 and study 302, NCT03056040). The first, a non-inferiority study of ALXN1210 compared to Eculizumab, directed to naive PNH patients with signs of hemolysis (defined as LDH values >1.5 times ULN), enrolled 246 subjects, randomized 1: 1 to receive either Ravulizumab (at the dose of 2700 +/- 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 P for non-inferiority <0.001). It has also been shown to have excellent safety and tolerability with the administration schedule every 8 weeks. The second, study 302, designed as a switch-study, was directed to PNH patients already being treated with standard dose of Eculizumab, in stable clinical conditions, to evaluate the non-inferiority of Ravulizumab. 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 confirmed non-inferiority also 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 FDA, while approval by the EMA is expected soon [119-125].

SKY59 / RO711268 / Crovalimab (Roche)

SKY59 is a recycling humanized anti-C5 monoclonal antibody with a long half-life, able to block C5 in a sustained and long-lasting way and, consequently the complement activity, even in Japanese patients carrying the gene variant (p.Arg855His) which makes them resistant to Eculizumab [126-127]. This molecule is currently being tested in a phase I/II study (NCT03157635), divided into 3 sequential parts and an open-label extension. In part 1, safety, tolerability, pharmacokinetics and pharmacodynamics were evaluated in healthy individuals, while in part 2 and 3, on patients in stable treatment with Eculizumab. The first data regarding the study have recently been published, showing that SKY59 is an effective C5 inhibitor, with excellent bioavailability 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 [128-130].

LFG316 (Novartis)

It is currently being tested in a phase I study (NCT025334909) in Japanese naive patients, aimed at evaluating the efficacy on carriers of the R885H polymorphism dependent on C5, which makes them resistant to Eculizumab therapy [131].

Coversin (Akari)

It is a 16 kDa protein derived from the Orinithodoros moubata tick and which inhibits the cleavage of C5 [132,133]. In the phase I study, in healthy volunteers, it showed good bioavailability after subcutaneous administration, with an excellent pharmacokinetic and pharmacodynamic profile, in the absence of immunogenetics or other safety issues. A first trial of efficacy was tested in Eculizumab-resistant patients, and subsequently, in the phase II study, Coversin was administered to PNH naive patients, in two daily subcutaneous doses (15-30 mg, after a loading dose of 60 mg) for 28 days, and then as a single dose (30 mg) for another two months. The drug 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: despite this, it would be advisable to improve the treatment regimen to optimize the response on intravascular hemolysis [134-138].

ALNCC5 (Alnylam)

ALNCC5 is a small interfering RNA, specific for C5, capable, in animal models, of completely silencing the production of C5 in the liver [139]. It was tested in healthy volunteers, to evaluate its efficacy and safety, and showed a reduction in plasma C5 levels (>99%) and an inhibition of the complement system (>95%). The phase I/II study (NCT02352493), apart from healthy volunteers, included an arm with six PNH patients. Patients received ALNCC5 at a weekly dose of 200 or 400 mg, 3 of them as monotherapy (PNH naive), and 3 of them in addition to Eculizumab therapy. No adverse events were observed, only one finding of a minimal rise in transaminases. The treatment presented different results in the two groups, although in all patients there was still a reduction in plasma C5 levels (>99%). In naive patients, in fact, the inhibition of C5 required about two months of treatment (an aspect not acceptable for patients requiring immediate treatment) and, in addition, a certain amount of intravascular hemolysis is present with LDH values >1.5 ULN. The best response is present in patients on combined treatment with normalization of LDH values. Based on these data, the best setting seems to be the combination therapy with Eculizumab, which could lead to a better control of intravascular hemolysis and/or a reduction in the dose of Eculizumab [140-143].

1.2.6 Therapeutic complement inhibition: the rationale for moving beyond anti-C5 agents

For many years, the cause of the greatest number of unsatisfactory responses to Eculizumab remained unclear. In this context, our group worked to understand what were the mechanisms underlying these failures and what solutions could be adopted.

The main cause of residual anemia in patients treated with Eculizumab is a C3-mediated extravascular hemolysis [109-144]. This phenomenon is present in all PNH patients treated with Eculizumab and is due to the continuous activation of the proximal portion of the complement system (mediated by the phenomenon of the C3 tick-over at the level of the alternative route), which is not affected by the blocking activity of Eculizumab, acting downstream on C5. This determines the accumulation of C3 fragments on PNH red blood cells, such as C3b through glycophorin A, for example. For this reason, the PNH RBCs are coated with fragments of C3, which as opsonins are recognized by the reticulo-endothelial system of the spleen and liver,

causing the phagocytosis of the coated red cells. This condition has been demonstrated in vivo with a reduction in the half-life of erythrocytes labeled with Chromium51 and, at the same time, showing an increased uptake of the radiocompound at the hepato-splenic level [109,144,145]. This mechanism, which affects the hematological response to Eculizumab in about 25-50% of patients, is however extremely different between subjects. It has been hypothesized that this variability may depend also on some hereditary polymorphisms involving complement regulatory genes that could predispose to a greater individual susceptibility to extra-vascular C3 hemolysis. Rondelli et al [146] have shown in fact that patients carrying a variant of the 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. There are currently no therapeutic strategies to "solve" C3-mediated extravascular hemolysis; corticosteroids have been used in the past, without success and with numerous side effects. Splenectomy has proved to be certainly useful but not decisive, therefore it is not considered a routine therapeutic choice [147-149].

The phenomenon of C3-mediated extravascular hemolysis has now been acknowledged as the main reason accounting for suboptimal hematological response to Eculizumab, and it has trigger the development of new treatment strategies for PNH, aiming to improve the results achieved with Eculizumab.

1.2.7 New inhibitors of the proximal portion of the complementary cascade

AMY-101 (Amyndas)

AMY-101, also known as Compstatin, is a peptide capable of binding C3 and C3b, preventing C3 cleavage and incorporation of C3b into C3/C5 covertase, and preventing activation of the complement system through its three ways (classical, alternative and lectin). AMY-101 is a next-generation form of Compstatin with a higher inhibitory capacity and a better pharmacokinetic profile. In vitro, this molecule blocks C3 and prevents erythrocyte opsonization; this could be useful for the control of both intravascular and extravascular hemolysis. A phase I study was conducted on healthy volunteers to evaluate their safety, tolerability, pharmacodynamics and pharmacokinetics. The first data announced seem encouraging; the molecule is well tolerated and has an excellent pharmacokinetic and pharmacodynamic profile with subcutaneous administration every 48 hours. Phase II study in naive PNH patients is planned [150-155].

APL-2 (Apellis)

It is the pegylated version of the first generation POT-4 , with a long duration of action. Two double-blind phase I studies were conducted in 40 healthy volunteers to evaluate their safety, tolerability, pharmacokinetic and pharmacodynamic profile. Twenty-four patients received a single dose (from 45 to 1440 mg subcutaneously), and the other 16 received multiple daily doses, again subcutaneously at a dose of 30 to 270 mg. There were no adverse events or treatment interruptions; plasma APL-2 concentration increases linearly over time reaching steady-state on day 28 of treatment. The blocking of the activity of the complement system is achieved either with a single dose of 1400 mg or with multiple doses of 180 or 270 mg. Two studies were conducted in PNH patients: PHAROAH (NCT02264639), in subjects treated with Eculizumab with inadequate response (defined by Hb <10g/dL and transfusion requirements); and the PADDOCK (NCT02588833) dedicated to naive PNH patients with significant hemolysis (defined by LDH values >2x ULN). APL-2 was administered subcutaneously, once a day, at a tapering dose of up to 270 mg. 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 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. Based on these data, Apellis designed a phase III study 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 [156-161].

LNP023 (Novartis)

LNP023 is a small oral Factor B inhibitor of the alternative pathway of the complement system. In vitro, it inhibits the lysis and opsonization of the red cells mediated by C3. The molecule is currently being studied, in a phase II trial, as an adjunct to Eculizumab in PNH patients with inadequate response (defined for LDH values > 1.5 ULN). The study aims 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 aim is to evaluate the improvement in hemolysis, estimated through the LDH values. Secondary aims include the evaluation of hemoglobin values, of the deposition of C3 on red blood cells [162-164].

ACH-4471 (Achillion)

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 [165-166]. The phase I study was conducted in healthy volunteers, who were administered with scalar doses; with 200-600 mg the plasma peak was reached, 1-2 hours after administration and with a half-life of the drug of about 9 hours. Ten naive PNH patients with signs of intravascular hemolysis were enrolled in the phase II study; final results are expected, patients are continuing therapy in the extension phase of the study. In parallel, there is also a phase II study 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 is to evaluate the increase in hemoglobin values [167-170].

The only treatment currently available for PNH patients is Eculizumab, some new drugs are currently in experimentation.

One of these, Danicopan (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 [165-166].

We investigated the FD inhibitor Danicopan as single-agent treatment for untreated PNH, aiming to control IVH while preventing C3-mediated EVH.

2.1 Background and rationale of the study

During AP initiation, the serine protease complement factor D (FD) cleaves factor B, leading to AP C3 convertase generation. Danicopan is a first-in-class, oral, small-molecule FD inhibitor that prevents new AP C3 convertase formation [175]. Consequently, proximal inhibition at the level of FD blocks AP-initiated upstream events and up to 80% of classical or lectin pathway initiated downstream events via amplification-loop inhibition 176. For PNH, targeting FD inhibition with a small molecule represents a potentially important treatment advancement because proximal AP inhibition may disable both terminal complement activation (inhibiting MAC–mediated IVH) and C3 fragment opsonization (preventing EVH), with additional convenience of oral administration.

The phase I study was conducted in healthy volunteers, who were administered with scalar doses; with 200-600 mg the plasma peak was reached, 1-2 hours after administration and with a half-life of the drug of about 9 hours. The development plan has continued with two phase 2 studies which enrolled different populations of PNH patients. The development of ACH-4471 is the subject of our work, which aimed to improve the current standard of care of PNH.

The first phase II study was conducted 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 is to evaluate the increase in hemoglobin values [161-170].

Phase 2 Study of Danicopan in Paroxysmal Nocturnal Hemoglobinuria Patients with an Inadequate Response to Eculizumab

Patients received oral Danicopan (ACH-4471; ACH-0144471; ALXN2040) at a 100 mg or 150 mg thrice daily starting dose and were instructed to take doses approximately the same time each day and as close as possible to 8 hours apart. Dose escalations, permitted at 4-week intervals to a maximum of 200 mg thrice daily in 50 mg increments, were based on safety and Hb levels through week 12. If the patient had not reached the 200 mg thrice daily maximal dose by week 12, escalation was permitted if clinically indicated.

Patients continued their pre-existing intravenous regimen of Eculizumab.

There was a mean Hb increase of 2.4 g/dL at week 24 (primary endpoint). This treatment effect appeared by week 2 in most patients and was maintained for the duration of the study.

The averages of the annualized rates of transfusion events were 5.2 pre-Danicopan and 0.2 post-Danicopan with a ratio (pre- vs post-Danicopan) of 0.04 (p=0.0009), demonstrating a highly statistically significant 95.9% reduction in transfusion frequency with Danicopan. The averages of the annualized transfusion units were 9.2 pre-Danicopan and 0.4 post-Danicopan with a ratio (pre- vs post-Danicopan) of 0.05 (p=0.0028).

There were substantial and significant ($p<0.05$ to $p<0.0001$) mean decreases from baseline to week 24 in absolute reticulocyte counts (from 219 to 135 10^9/μL), in total bilirubin (from 2.17 to 1.35 mg/dL) and in direct bilirubin (from 0.51 to 0.37 mg/dL). No change in the mean LDH relative to the upper limit of normal was also observed (from 1.06 at baseline to 1.04 x ULN at week 24).

FACIT-Fatigue scores were reported, with a mean increase of 11 points at week 24 from baseline score of 34 on Eculizumab alone [171].

Consistent with the well-established data on C3 fragment opsonization in PNH patients treated with Eculizumab [99,109,114,172,173], the GPI-deficient erythrocytes opsonized with C3 fragments (i.e., C3d+ GPI-deficient erythrocytes) were readily detected at baseline with a mean value of 22% as a consequence of C3 fragment accumulation on cells having survived IVH in the presence of a C5 inhibitor [99,109]. The addition of Danicopan significantly decreased the percentage of GPI-deficient erythrocytes opsonized with C3 fragments (mean 6.7% at week 24). Concomitantly, the clone size of GPI-deficient erythrocytes increased from 54% at baseline to 84% (mean) at week 24, approaching the clone size of the GPI-deficient granulocytes (mean: 92% at baseline and 95% at week 24) [171].

The efficacy in the combination study has led to the use of Danicopan in monotherapy in naïve patients, to evaluate the use of an only oral therapy in a subsetting of patients who otherwise would be obliged to access hospital to carry out IV therapy every two weeks.

The second study was instead conducted in untreated PNH patients, and represents our original work.

This study aims to:

1. Evaluate the hematological response to treatment with Danicopan monotherapy in naive patients and monitor the safety and perceived quality of life

2. Investigate in vivo pathophysiological changes on Danicopan (C3 mediated extravascular hemolysis, changes in PNH clone size).

The parameters analyzed at 2 time points (4 and 12 weeks of treatment) were

1. the concentration of Hb

2. the LDH values

3. laboratory parameters, indicators of residual hemolysis, such as reticulocyte count, haptoglobin, total bilirubin.

4. Bb fragment and deposition of C3 on red blood cells

5. the size of the PNH clone

3.1 Patients and treatment

This study was conducted from March 2017 to November 2018. After this period an extension study was started, which was offered to all patients enrolled in the initial study (Phase 2, Open-label, Multi-Center, Multi-Dose, Extension).

Each patient was evaluated in collaboration with belonging Center on the basis of all clinical and laboratory information; furthermore, biological samples were collected for each patient for the centralized experimental study, biological samples were analyzed locally for the patients we treated for complementary analyzes.

3.2 Key inclusion and exclusion criteria

We enrolled adult (≥ 18 years old), untreated PNH patients with hemoglobin <12 g/dL (and adequate reticulocytosis per investigator), GPI-deficient granulocyte or Type III erythrocyte clone size ≥10%, lactate dehydrogenase (LDH) ≥1.5 times upper limit of normal (ULN), platelets ≥50,000 µL, and that were vaccinated for N. Meningitidis, H. Influenzae, and S. Pneumoniae.

None of the subjects were receiving Eculizumab <75 days before study due to lack of availability and/or patient willingness and none had history of organ, stem cell or marrow transplant.

3.3 Trial

Patients received oral Danicopan at a starting dose of 100 mg or 150 mg, thrice daily. Dose escalations were permitted based on hemolysis control, assessed by LDH, per investigator assessment in stepwise increments up to 200 mg thrice daily. Doses were to be taken at approximately the same time each day and as close as possible to 8 hours apart. All doses should be taken approximately 15-30 minutes after completion of a meal or snack. Treatment duration was 84 days; patients completing treatment with clinical benefit entered a long-term extension (figure 7).
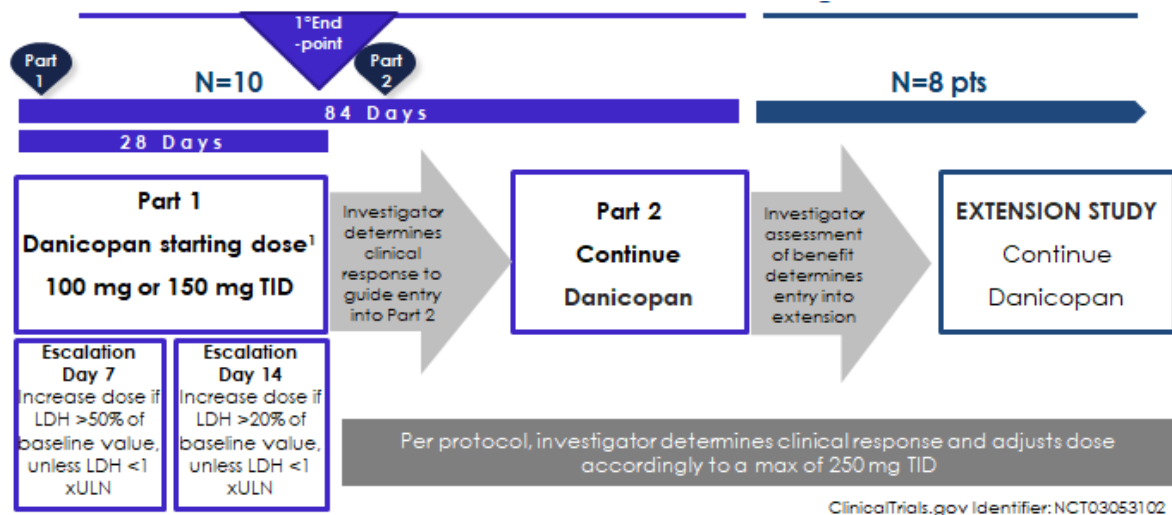
Figure 7. Clinical Trial design

3.4 Follow-up procedures

At each visit the patient 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 Danicopan.

3.5 Statistical analysis

Due to the small sample size, only descriptive and exploratory statistics were utilized to present results for continuous biochemical and quality-of-life measurements. Discontinued patients were not replaced. Missing values were not imputed. To summarize categorical data, frequency counts and percentages are presented. The Pearson correlation coefficient (Pearson's r) is used to examine the relationship between two variables.

4.1 Patient characteristics

Eleven patients were screened. Ten untreated patients with hemolytic PNH were enrolled and received Danicopan. Of these patients 2 were directly followed and evaluated at the AOU Federico II.

Median age was 33 years (range 17-63 years) and median disease duration was 5.9 years (range 0-14 years). Mean GPI-deficient clone size was 79% for granulocytes and 32% for erythrocytes, LDH levels (1416±540 U/L, corresponding to 5.7±2.17 times ULN), increased reticulocyte count (154±69×10^9/L), increased total bilirubin (1.3±0.74 mg/dL), and reduced haptoglobin (5.8±2.9 mg/dL). Mean baseline hemoglobin was heterogeneous among patients (9.8±1.8 g/dL) and four of ten patients had a transfusion history with a median of 14.5 red blood cell units in the three years before dosing (table 1).

	MEAN	MEDIAN	RANGE
Age		33 years	17-63 years
Disease duration		5,9 years	0-17 years
Erythrocytes GPI-deficient	32%		
Granulocytes GPI-deficient	79%		
LDH levels	1416±540 U/L		
Reticulocyte count	154±69*10^9/L		
Total bilirubin	1.3±0.74 mg/dL		
Haptoglobin	5.8±2.9 mg/dL		
Hemoglobin	9.8±1.8 g/dL		
Red blood cell units/3 years		14,5	

Table 1. Baseline characteristics of the patients

4.2 Study disposition and safety

Two patients started Danicopan at 100 mg thrice daily and increased to 150 mg thrice daily, and eight started 150 mg thrice daily. Increases to 175 mg and 200 mg thrice daily were performed in eight and four patients, respectively. All 10 patients reached day 28 and are included in the primary endpoint evaluation. Two discontinued before day 84: one for a serious adverse event of elevated aspartate aminotransferase/alanine aminotransferase

coincident with breakthrough hemolysis, which resolved without sequelae; the other withdrew for personal reasons unrelated to safety. All patients were evaluated until they left the study or reached day 84 (N=8). Nine patients (90%) developed at least one adverse event during treatment; only one was serious. With few exceptions, adverse events were mild and resolved during the study. There were no clinically significant changes to other key laboratory parameters during treatment (table 2).

		% of total	Standard severity grade			
Primary system organ class preferred term*	n	(N=10)	Mild	Moderate	Severe	Life-threatening
Number of subjects reporting	9	90	8	1		
Number of unique TEAEs†	33	NA	26	4	2	1
Number of subjects with SAEs	1	10	..	..	1	..
Blood and lymphatic system disorders	2	20	..	..	..	..
Hemolysis	2	20	1	..	1	..
Gastrointestinal disorders	3	30	..	..	..	..
Abdominal pain	1	10	..	1	..	..
Mouth ulceration	1	10	1	..	..	..
Nausea	1	10	1	..	..	..
Vomiting	1	10	1	..	..	..
General disorders and administration site	4	40	..	..	..	..
Fatigue	1	10	1	..	..	..
Non-cardiac chest pain	1	10	1	..	..	..
Edema, peripheral	1	10	1	..	..	..
Vaccination site erythema	1	10	1	..	..	..
Infections and infestations	5	50	..	..	..	..
Pharyngitis	1	10	..	1	..	..
Upper respiratory tract infection	4	40	3	1	..	..
Viral upper respiratory tract infection	1	10	1	..	..	..
Injury, poisoning, and procedural complications	1	10	..	..	..	..
Contusion	1	10	1	..	..	..
Investigations	1	10	..	..	..	..
Alanine aminotransferase increased	1	10	..	..	1	..
Aspartate aminotransferase increased	1	10	..	..	..	1
Metabolism and nutrition disorders	1	10	..	..	..	..
Iron deficiency	1	10	1	..	..	..
Musculoskeletal and connective tissue	3	30	..	..	..	..
Back pain	2	20	2	..	..	..
Myalgia	1	10	1	..	..	..
Nervous system disorders	4	40	..	..	..	..
Headache	4	40	4	..	..	..
Psychiatric disorders	1	10	..	..	..	..
Irritability	1	10	1	..	..	..
Renal and urinary disorders	3	10	..	..	..	..
Hemoglobinuria	2	20	2	..	..	..
Paroxysmal nocturnal hemoglobinuria	1	10	..	1	..	..
Reproductive system and breast disorders	1	10	..	..	..	..
Dysmenorrhea	1	10	1	..	..	..
Skin and subcutaneous tissue disorders	1	10	..	..	..	..
Rash, papular	1	10	1	..	..	..

Table 2. *MedDRA Version 18.1; †The row represents the number of events; all other rows represent the number of subjects; NA=not available; SAEs=serious adverse events; TEAEs=treatment-emergent adverse events.

4.3 Efficacy

We considered LDH at day 28 the primary endpoint and we observed a significant reduction among all 10 patients from a mean value of 5.7±2.17 times ULN at baseline to 1.8±1.03 times ULN at day 28 (p<0.001). The percentages of patients showing LDH <3 times ULN, <2 times ULN, and <1 time the ULN were 90%, 60%, and 40%, respectively.

The secondary endpoints were LDH at day 56 and 84, there was a reduction from baseline of respectively 2.3±1.41 times ULN (p<0.005) and 2.2±1.04 times ULN (p<0.001). The percentages of patients showing LDH <3 times ULN, <2 times ULN, and <1 times ULN were 71%, 43%, and 43% at day 56, and 75%, 37.5%, and 25% at day 84, respectively (figure 8). Nevertheless, only two breakthrough hemolytic events were recorded by the investigator as adverse events a third patient experienced recurrent subclinical breakthrough episodes as a consequence of inadequate control of complement activation.
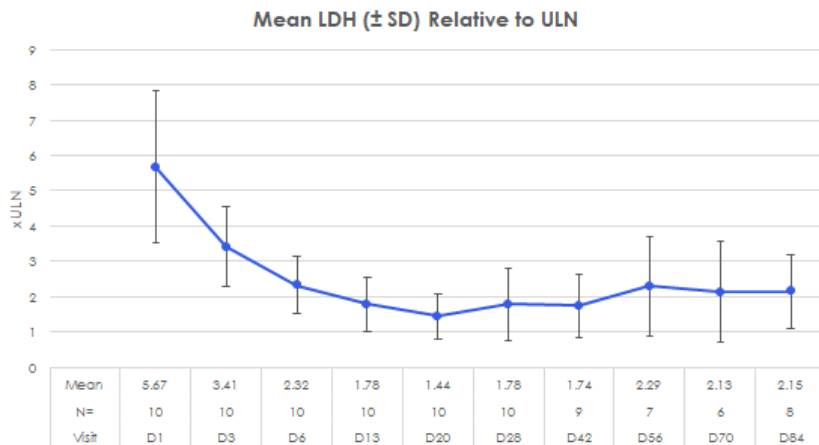

Mean LDH (± SD) Relative to ULN

	D1	D3	D6	D13	D20	D28	D42	D56	D70	D84
Mean	5.67	3.41	2.32	1.78	1.44	1.78	1.74	2.29	2.13	2.15
N=	10	10	10	10	10	10	9	7	6	8

Figure 8. Effect of Danicopan on LDH

It was observed an increase of Hemoglobin mean (secondary endpoint) from 9.8 g/dL at baseline (range 6.9 g/dL to 12.0 g/dL) to 10.9 g/dL at day 28 (range 8.4 to 14.1; p<0.005), 10.9 g/dL at day 56 (range 8.5 to 13.1; p<0.005), and 11.5 g/dL at day 84 (range, 8.7 to 13.7, p<0.005). Mean increase versus baseline was 0.9 g/dL at day 28 and 1.7 g/dL at day 84 (figure 9).

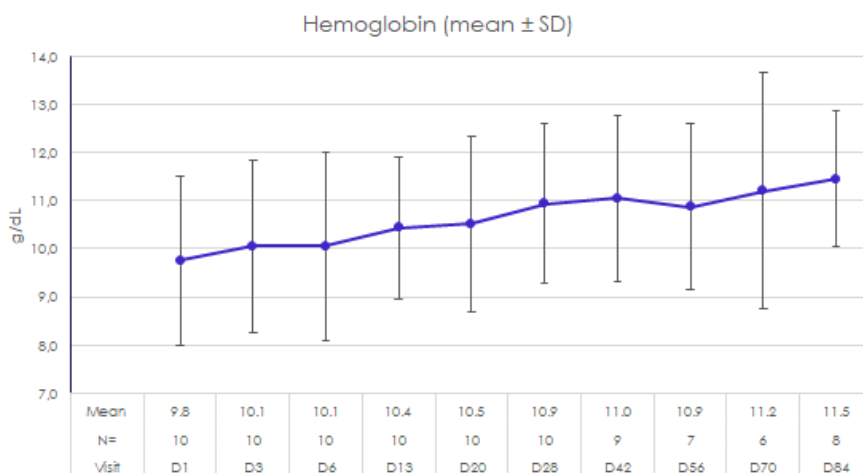|										
Mean	9.8	10.1	10.1	10.4	10.5	10.9	11.0	10.9	11.2	11.5
N=	10	10	10	10	10	10	9	7	6	8
Visit	D1	D3	D6	D13	D20	D28	D42	D56	D70	D84

Figure 9. Effect of Danicopan on Hemoglobin

There was also an increase of FACIT–Fatigue score mean (secondary endpoint): at baseline was 34 and increased by 9 and 13 points was seen at days 28 and day 84, respectively (p<0.05).

There was a percentage increase of 56±19.9% at day 84 vs 32±24.6% at baseline (p=0.001) of GPI-deficient erythrocytes (secondary endpoint), whereas no change was observed for GPI-deficient granulocytes.

It was observed a decrease of total bilirubin (secondary endpoint) after Danicopan treatment (0.6±0.23 mg/dL at day 84 vs 1.3±0·74 mg/dL at baseline, p<0.05) and an increase of haptoglobin (secondary endpoint): 15.3±16.08 mg/dL at day 84 vs 5.8±2.89 mg/dL at baseline (p=0.15) but the was no statistical significativity on the contrary of absolute reticulocyte count (secondary endpoint) that decreased quickly and was sustained with treatment ($81±33.6×10^9$/L at day 84 vs $154±69×10^9$/L at baseline, p<0.05).

Bb fragment, an activation product of factor B, tracks complement AP activation in vivo. Plasma Bb level was significantly elevated at baseline (2.24±0.77 µg/mL) compared with healthy volunteers (0.84±0.212 µg/mL; p<0.05). After Danicopan, Bb level was significantly reduced: day 28, 0.84±0.84 µg/mL (p<0.005); day 56, 0.47±0.09 µg/mL (p<0.005); day 84, 0.47±0.06 µg/mL (p<0.005). In contrast to residual AP activity, Bb level remained consistently low irrespective of subtherapeutic plasma Danicopan levels around predose periods. A strong positive correlation was found between Bb and LDH (Pearson's r=0.80, p<0.0001), supporting Bb as a reliable biomarker of in vivo AP activation in PNH and, therefore, its value for monitoring efficacy. Danicopan also showed strong linear correlations

with Bb and LDH (negative), as did AP with Bb and LDH (positive); this validated the role of Danicopan in AP inhibition and subsequent in vivo changes of Bb and LDH (figure 10).
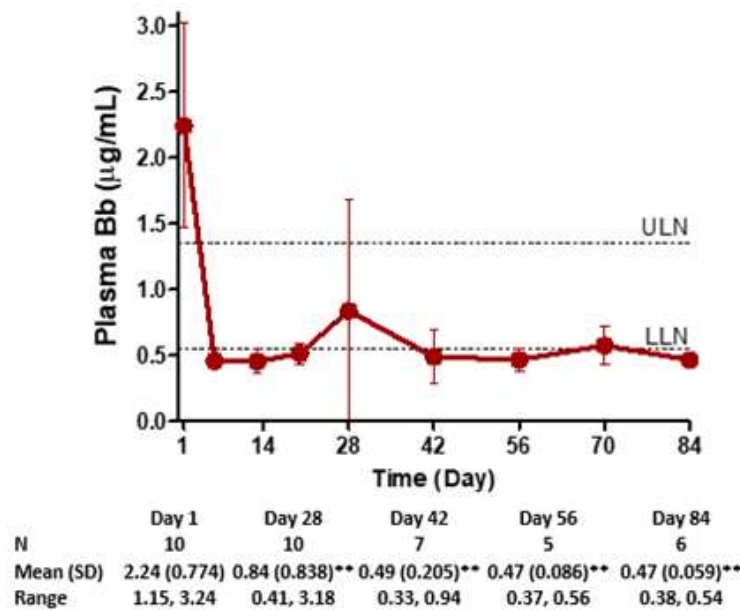

	Day 1	Day 28	Day 42	Day 56	Day 84
N	10	10	7	5	6
Mean (SD)	2.24 (0.774)	0.84 (0.838)**	0.49 (0.205)**	0.47 (0.086)**	0.47 (0.059)**
Range	1.15, 3.24	0.41, 3.18	0.33, 0.94	0.37, 0.56	0.38, 0.54

Figure 10. Plasma Bb concentration (mean ± SD) at baseline (day 1) through the end of the study (day 84) with descriptive statistics. The dashed lines represent ULN and LLN which are derived from phase 1 studies in healthy volunteers (see Assay Methods in Supplement). N values of <10 for plasma Bb on days 42, 56, and 84 reflect the two early discontinuations and additional samples not collected. **p<0.005. Note, LLN=lower limit of normal. SD=standard deviation. TID=thrice daily. ULN=upper limit of normal.

The analysis on Serum C3 showed that there was an increase of it (114.2 ± 17.3 mg/dL at day 84 vs 102.2 ± 20.2 mg/dL at baseline, p=0.08), likely from reduced C3 consumption because of upstream complement blockade. Importantly, C3 fragment deposition on erythrocytes was very low (<0.5% of erythrocytes) throughout treatment [174].

4.4 Index patients

We report here detailed results of two index patients who were enrolled at our site; they are paradigmatic of the two typical clinical scenarios observed during Danicopan treatment.

Patient A is a 28.5-year-old female with PNH from 7 years and 1 month, who has not received any etiologic anti-complement treatment until the time of enrollment. Prior to treatment, the mean size of GPI-deficient clones was 96% for granulocytes and 29.9% for erythrocytes. Overt hemolysis was demonstrated by elevated LDH levels (7.45 times ULN, maximum 14.83 times ULN, minimum 2.91 times ULN), increased reticulocyte count

$(200*10^9/L$, maximum $409*10^9/L$, minimum value $115*10^9/L)$, increase in total bilirubin (1.94 mg/dL, maximum value 3 mg/dL, minimum value 1.26 mg/dL) and reduced haptoglobin (0.07 g/L). The mean hemoglobin at baseline was 9.8 g/dL (maximum value 12.6 g/dL minimum value 7.7 g/dL) and she has never had a blood transfusion. She have had frequent episodes of hemolysis resulting in asthenia and hemoglobinuria. She has never had thrombosis.

Patient B is a 30.9-year-old male with PNH for 10 years and 2 months, who has not received any etiologic anti-complement treatment until the time of enrollment. Prior to treatment, the mean size of GPI-deficient clones was 64% for granulocytes and 18.78% for erythrocytes. Manifest hemolysis was demonstrated by elevated LDH levels (3.48 times ULN, maximum 5.88 times ULN, minimum 3.05 times ULN), increased reticulocyte count $(102*10^9/L$, maximum $150*10^9/L$, minimum value $88*10^9/L)$, increase in total bilirubin (1.23 mg/dL, maximum value 1.56 mg/dL, minimum value 0.67 mg/dL) and reduced haptoglobin (0.07 g/L). The mean hemoglobin at baseline was 11.2 g/dL (maximum value 13.6 g dL minimum value 9 g/dL) and he has never had a blood transfusion. He have had frequent episodes of hemolysis resulting in asthenia, muscle cramps with increased creatine phosphokinase and transaminases and hemoglobinuria. He has never had thrombosis.

During their disease course, both patients were considered candidate to start standard anti-complement treatment with Eculizumab. However, their clinical presentation was deemed not severe enough to oblige physician to start Eculizumab, even because both patients prefer to not start a life-long chronic treatment requiring frequent hospitalization likely impacting on their daily life. Patients were enrolled in this trial because it was assumed that Danicopan was a good drug to control hemolytic crises (patients with high LDH and medium hemoglobin compensation) at least improving their clinical presentation, while preserving their life-style of young active adults (oral administration).

Our two patients reached the primary and the secondary endpoints with a good control of intravascular and extravascular hemolysis demonstrated to the increase of hemoglobin and decrease of LDH, bilirubin and reticulocytes (table 3)

	Patient A			Patient B		
	BL	D28	D84	BL	D28	D84
Danicopan (mg po TID)	150	175	175	150	175	200
Hgb (g/dL)	9.5	10.2	11.6	10.0	11.4	11.9
LDH (xULN)	8.55	1.51	3.26	6.08	2.29	2.98
Reticulocytes (10^9/μL)	217	74	48	171	78	118
Total bilirubin (mg/dL)	1.88	0.88	0.74	1.32	0.54	0.71
GPI-deficient RBC clone size (%)	21	47	59	13	35	42
FACIT-Fatigue†	44	49	50	39	46	46

Table 3. Baseline Characteristics and Clinical Results. BL- baseline; D- day; Hb- hemoglobin; FACIT-functional assessment of chronic illness therapy; GPI- glycosylphosphatidylinositol; LDH-lactic acid dehydrogenase; po- orally; RBCs- red blood cells; TID- three times a day; ULN- upper limit of normal

Since both patients achieved the efficacy endpoint of the study (figure 11) with major clinical benefit, they both were subsequently enrolled in the extension study which was offered to all patients participating to the initial study (Phase 2, Open-label, Multi-Center, Multi-Dose, Extension). Here we show for these two patients a follow up as long as 30 months.
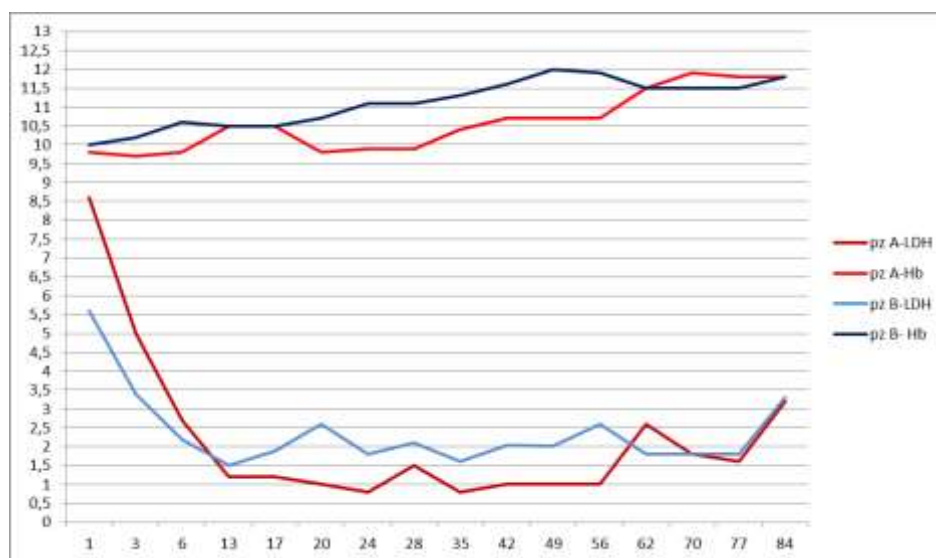

Figure 11. Patient A and B: effect of Danicopan on LDH (times ULN) and hemoglobin (g/dL) from day 1 to day 84

Patient A after 30 months from the beginning of the trial had no adverse event, no infection events, and no thrombosis, showing a better control of hemolysis with lower LDH levels (1.62 times ULN, maximum 8.63 times ULN, minimum value 0.8 times ULN), reticulocyte count (73*10^9/L, maximum 227*10^9/L, minimum value 53*10^9/L) and total bilirubin (0.7 mg/dL, maximum 2.3 mg/dL, minimum value 0.4 mg/dL). Mean hemoglobin was 12.05 g/dL (maximum 13.6 gr/dL with some fluctuations but always higher than 12 after 6 months from

the start of the trial) she had a good quality of life (reduced asthenia and symptoms of hemolysis) with some episodes of subclinical hemolysis (figure 12).

Figure 12. Patient A: effect of Danicopan on LDH and hemoglobin in the in the extension trial

During the treatment, since PNH red cells were better protected from hemolysis, the proportion of PNH cells on the red blood cells increased (after six months of treatment always above 71.63% also reaching the maximum value of 86.42%) (figure 13), while the PNH clone remained constant for white blood cells and monocyte.
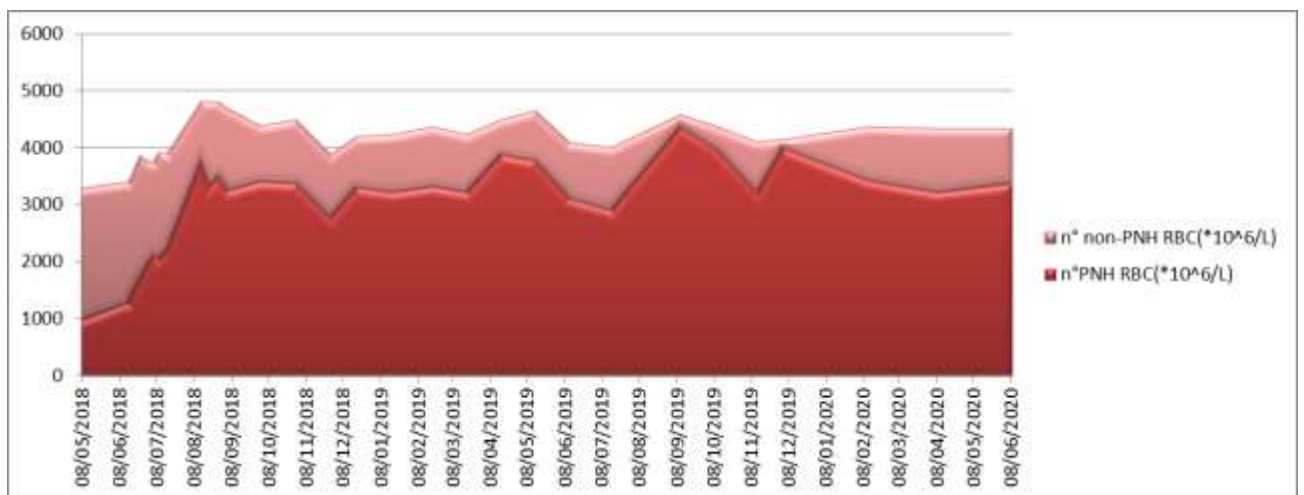

Figure 13. Patient A: number of RBCs with PNH phenotype and with no PNH phenotype in the extension trial

In the first 6 months of trial there was a rapid decrease of LDH with a slower rise of hemoglobin value with a normalization of value of hemoglobin with some intercurrent subclinical breakthrough hemolysis with no evidence of infection episodes.

Patient A had a good response to Danicopan with minimal episodes of subclinical hemolysis.

Similarly, patient B had no adverse events, no infection events and no thrombosis, achieving a better control of hemolysis with lower LDH levels (2.54 times ULN, maximum 5.59 times ULN, minimum value 1.38 times ULN), reticulocyte count ($96*10^9$/L, maximum $206*10^9$/L, minimum value $75*10^9$/L) and total bilirubin (0.9 mg/dL, maximum 2.7 mg/dL, minimum value 0.2 mg/dL). Mean hemoglobin was 12.1 g/dL (maximum 14 gr/dL with some fluctuations but always higher than 12 after 15 months from the start of the trial) (figure 14). He had a good quality of life with some episodes of subclinical hemolysis.
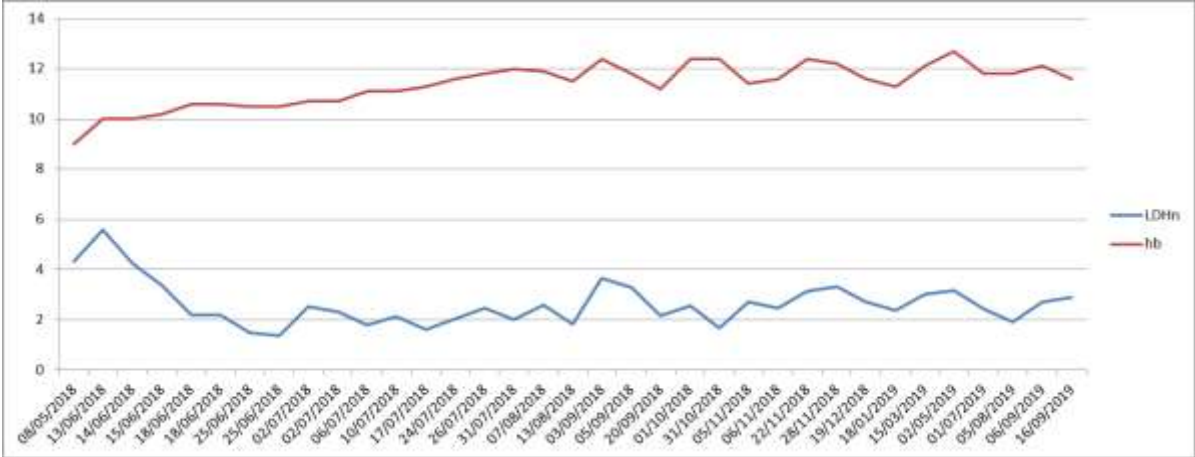

Figure 14. Patient B: effect of Danicopan on LDH and hemoglobin in the in the extension trial

During the treatment the proportion of PNH cells on the red blood cells increased but with consistent fluctuations: the mean of the clone was 44.25% but with maximum peaks of 67.4% and minimums of 39.4% (figure 15).
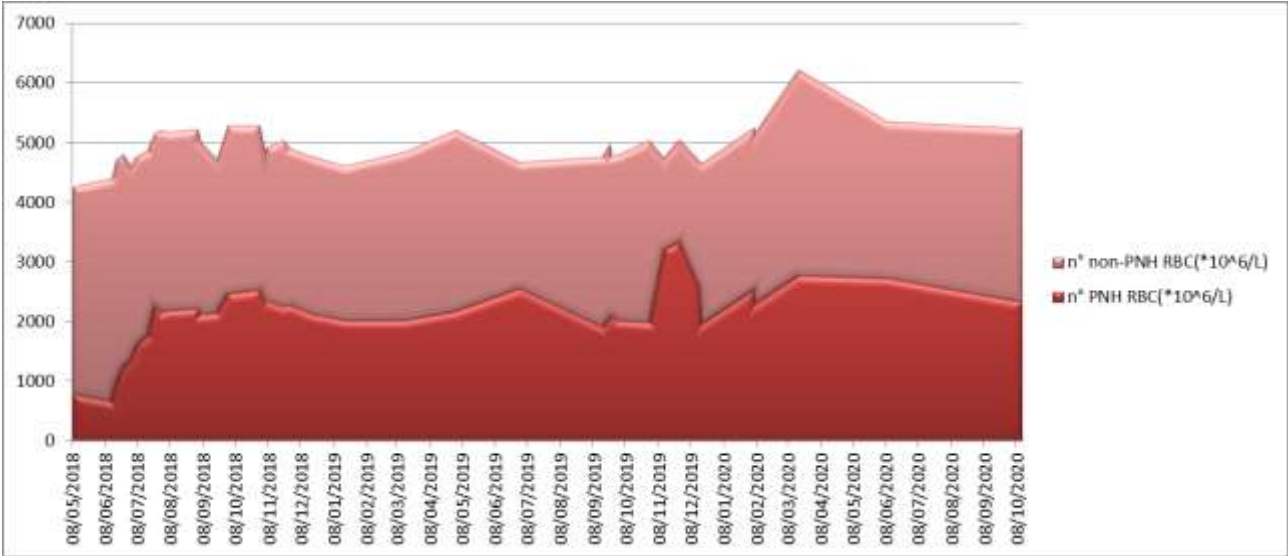

Figure 15. Patient B: number of RBCs with PNH phenotype and with no PNH phenotype in the extension trial

For this second patient B, the time to reach a normal value of hemoglobin was longer (15 months vs. 6 of patient A); in addition, even if LDH showed a significant decrease, it continued to exhibit significant fluctuations, with transient increases associated with intercurrent subclinical breakthrough hemolysis with no evidence of infection episodes. This was associated with the persistence of a higher proportion of non-PNH erythrocytes, eventually suggesting that some residual hemolysis remained irrespective of the Danicopan treatment.

Patient B had good response to Danicopan but retaining a continuous residual hemolysis sometimes with paroxysmal seizures.

Anti-complement therapy with the anti-C5 agent Eculizumab has revolutionized the treatment and prognosis of patients with paroxysmal nocturnal hemoglobinuria, replacing supportive therapies, which are ineffective in prolonging their long-term survival. However, unmet clinical needs remain even in the Eculizumab era, such as residual intravascular hemolysis, breakthrough episodes and mostly extravascular hemolysis, which emerges in most PNH patients on anti-C5 therapy due to the continuous activation of the complement alternative pathway, through the phenomenon of the C3 tick-over. The continuous activation of the proximal complement, irrespective of a sustained blockade of the terminal complement, leads to the accumulation of C3 fragments on PNH red blood cells, which serves as opsonins favouring the removal of these erythrocytes by tissue macrophages in the spleen and in the liver. C3-mediated extravascular hemolysis, together with residual intravascular hemolysis and possible bone marrow failure, are the pathogenetic mechanisms accounting for residual anemia in most PNH patients treated with Eculizumab.

Clinical development of proximal complement inhibitors has been motivated by the description of C3-mediated EVH as a mechanism driving significant anemia and limiting hematologic benefit in some PNH patients on Eculizumab and other C5 inhibitors [109,177]. Proximal inhibitors were initially conceived to prevent C3-mediarted extravascular hemolysis which emerges during anti-C5 therapies; thus, they were developed to improve the efficacy of anti-C5 agents, possibly in a combination treatment. However, it has been hypothesized that proximal inhibitors, by preventing generation of downstream C5 convertases, can be effective even in absence of terminal inhibitors (i.e. Eculizumab or other C5 inhibitors). Indeed, by disabling the initiating event of complement activation, Danicopan and other proximal inhibitors should prevent the generation of C5 convertases, obviating the need for downstream C5 inhibition. Additionally, specific targeting of AP can preserve classical and lectin pathway–mediated anti-microbial activity.

Here we investigated Danicopan, a first-in-class oral FD inhibitor, which blocks the proximal complement cascade upstream of C5 at the level of AP initiation and amplification. In untreated PNH patients, Danicopan used in monotherapy resulted in inhibition of IVH, with significant LDH reduction at day 28 (primary endpoint) and throughout treatment duration. As anticipated by its mechanism of action, Danicopan also prevented C3 deposition on

surviving GPI-deficient erythrocytes, preventing EVH (confirmed by reduction of bilirubin and reticulocytes). Concomitant inhibition of IVH and prevention of C3-mediated EVH resulted in improvement of anemia, with a mean hemoglobin gain of 1.7 g/dL after 12 weeks of treatment. Consistent with these findings, all patients exhibited significant increases in the percentage of GPI-deficient erythrocytes, confirming their extended half-life in vivo, and improvement in FACIT-Fatigue quality-of-life measurements. With the caveat of limited sample size and exposure, no safety concerns or infectious complications emerged during the study other than those described.

The clinical effects observed were achieved irrespective of a low-level residual IVH, which remained detectable in some patients (fluctuations in LDH). This residual IVH is likely the consequence of the increase of GPI-deficient erythrocytes susceptible to complement-mediated hemolysis and the possible transient exacerbations, this was due to transient weaker AP inhibition around Danicopan predose periods [174]. In other words, while this proof-of-concept study clearly demonstrates that interception of the complement cascade at the level of FD efficiently protects PNH erythrocytes from MAC-mediated intravascular hemolysis and prevents their C3-mediated extravascular removal, it also shows that such inhibition needs to be complete and sustained to maximize the therapeutic effect.

Indeed, in the use of Danicopan has shown an improvement in LDH and hemoglobin values both in patients with insufficient response to Eculizumab and in untreated patients. Nevertheless, most patients did not achieve a full normalization of their hematological parameters, eventually highlighting some limits of the molecule.

Although the therapeutic strategy of blocking upstream of the complement cascade turns out to be effective and practicable, Danicopan does not perform this mechanism in the best way, mostly due to its pharmacokinetics and pharmacodynamics which may preclude AP inhibition >90% (the threshold of inhibition whish seems essential for clinical activity) being sustained continuously irrespective of administrations any 8 hours (the half-life of the the compound is pretty short) (figure 16).
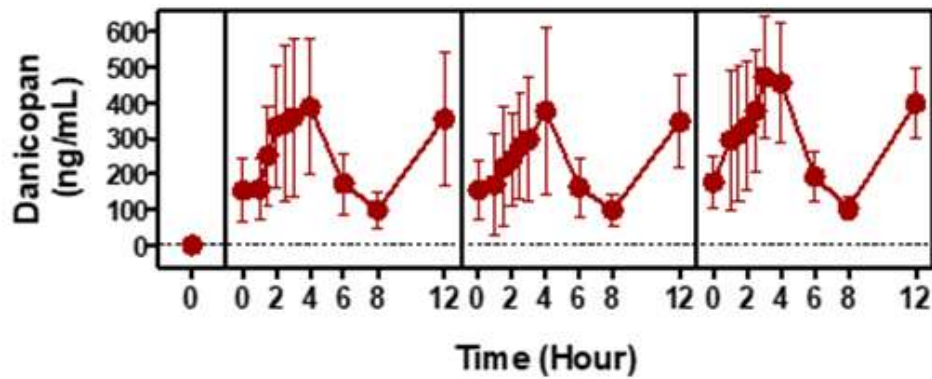
Figure 16. The mean ± SD of ex vivo plasma danicopan concentration, combining all dosing groups together.

This unfavorable pharmacokinetic profile may account for the recurrence of basal hemolysis levels transiently during the chronic treatment, especially in the few hours before the next dose, and/or in case of events triggering the complement activation (such as infections). Indeed, the risk of breakthrough hemolysis is even more relevant due to the efficacy of the treatment itself, which allows a much longer survival of PNH red blood cells eventually resulting in the circulation of much larger numbers of PNH red blood cells susceptible to complement mediated hemolysis, even suddenly, if the threshold of AP inhibition is transiently lost.

Therefore, perfect adherence to therapy by the patient becomes fundamental at the expense of greater manageability (inability to take the dose, health conditions that limit its absorption or prevent its administration such as episodes of vomiting become serious problems for plasma concentration of drug).

PNH is a disease due to AP disregulation (i.e. continuous, spontaneous C3 tick-over, eventually exacerbated at time of additional complement activation). The ideal drug for PNH should be able to completely disable the complement cascade at the level of C3 or even higher. It is only by delivering a full and sustained inhibition of proximal and terminal complement that the treatment may accomplish with all its goals, which (in comparison to Eculizumab) are:

1.   Improve control of intravascular hemolysis
2.   Prevent C3-mediated extravascular hemolysis
3.   Keep (or improve) prevention of thromboembolisms
4.   Normalization of hemoglobin, LDH, reticulocyte counts
5.   Prevent breakthrough hemolysis (both PK and PD) and breakthrough thromboembolisms

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6.   not expose patient to infectious risk

Preliminary data using Danicopan seem to address all these points, with the caveat of the intrinsic limitations of the compound in terms of pharmacokinetic and pharmacodynamics. Similar data have been generated even with other proximal inhibitors, such as the oral FB inhibitors or the subcutaneous anti-C3 agents.
All of these approaches look promising for the treatment of PNH and clinical data should tell us very soon what are the viable treatment options are in terms of safety and efficacy, and how we can best utilize in the appropriate patients (i.e., monotherapy vs. add-on treatment).

In conclusion, this study demonstrates that Danicopan appears to be well-tolerated and showed clinically meaningful IVH inhibition and hemoglobin improvement in untreated PNH patients with an easier mode of administration. Nevertheless, some unmet clinical needs remain, namely the residual intravascular hemolysis which may appear because the block of the complement cascade is not complete, exposing patients to pharmacokinetics or pharmacodynamics breakthrough hemolysis.

We can conclude that Danicopan is a good oral, proximal inhibitor of complement cascade which can be used in monotherapy for the treatment of PNH; however, it is not the perfect drug yet. Better efficacy is expected with next-generation agents, which combine a more favorable pharmacokinetics (they can be given twice a day due to longer half-life) with a deeper inhibition of FD. The novel FD inhibitor ACH-5228 has already started its development in a phase 2 study in PNH, showing potency three times superior to Danicopan and with a twice daily administration allowing sustained >90% AP inhibition in chronic treatment. Irrespective of the specific molecule, this strategy of proximal complement inhibition at the level of FD (as well as at the level of FB or of C3) promises to change the treatment paradigm of PNH in the next few years, eventually resulting in better hematological efficacy and more convenient treatment for patients.

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