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"*A TRANSLATIONAL INVESTIGATION ON UNMET CLINICAL NEEDS IN PNH"*

Settore scientifico-disciplinare: MED/15

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1. Introduction

1.1. Definition

PNH (PNH) is an ultra-rare, nonmalignant, blood disorder. PNH is conventionally included in the large class of hemolytic anemia, but its clinical features are really peculiar:

1.1) complement-mediated chronic intravascular hemolysis with acute crisis;

1.2) thrombotic events, most of the time venous and in atypical sites;

1.3) bone marrow failure that manifests by cytopenia/s or pancytopenia, depending on the numbers of blood cell lines involved [white blood cells, red blood cells (RBCs) and platelets].

This triad makes PNH a unique clinical syndrome among the other hemolytic disorders.

With regard to the terminology, the expression “Paroxysmale Hämoglobinurie” was coined for the first time in 1882 by Paul Strübing, to describe a case of an acquired hemolytic anemia with particular characteristics. Moreover, the adjective “Nocturnal” was added later, but then it was proven that PNH’s clinical manifestations can occur at any time of day or night [Luzzatto, 2006].

1.2. Historical background

In 1938, the groups of Thomas Hale Ham in Boston [Ham TH, 1938], John Dacie in London [Dacie JV, 1938] and F.L.J. Jordan in Utrecht [Jordan FLJ, 1938], found that PNH red blood cells were lysed when incubated with normal acidified serum and this phenomenon was abrogated by heating the serum or removing divalent cations, both reactions that reduce complement activity. More specifically, in these experiments the red blood cells were incubated with an excess of anti-I antibodies and this would also explain the lysis of some normal blood cells.

In a PNH patient, total RBC comprise three main populations: "PNH I" defined as normal, RBC lysate by just 1/25 of the serum concentration required to lysate normal RBC were defined as "PNH III" and those with an intermediate sensitivity were called “PNH II” [Rosse WF, 1974]. PNH II and PNH III populations of RBC vary in percentage from 1% to nearly 100%.

Later, Nicholson-Weller's team discovered that PNH RBC lacked the decay accelerator factor (DAF, later called CD55), which is normally present on the RBCl membrane and is intended to destroy the convertase complex [Nicholson-Weller A, 1983]. Glycosylphosphatidylinositol (GPI) anchor has the function of binding numerous proteins to the outer membrane of all cells [reviewed in Lowe MG, 1989], and in the 1980s it became clear that PNH cells were missing of all GPI-linked, including CD55 [Davitz MA, 1986]. Subsequently, Kinoshita's group demonstrated that the mutation of the PIGA gene on the short arm of the X chromosome and also of PIGT, PIGB and PIGV are the cause of PNH [Takeda J, 1993]. Finally, Parker's group discovered that in addition to CD55, also CD59 or "membrane inhibitor of reactive lysis" (MIRL) which under normal conditions controls the efficacy of the membrane attachment complex which consisted of C5b, C6 C7 C8 and C9 [Holguin MH, 1989] was absent on the PNH red blood cell membrane [Holguin MH, 1989].

Before flow cytometry, it had been shown that several molecules were absent only on PNH red blood cells: for example, acetylcholinesterase [Ghiotto & De Sandre,

1956; Auditore & Hartmann, 1960]. With the introduction of flow cytometry, it was found that a huge number of other proteins were missing not only on PNH red blood cells, but also on PNH granulocytes, platelets, monocytes and lymphocytes. In June 2007, eculizumab (ECU) became the first human chimeric monoclonal antibody to receive approval from the European Commission under the accelerated assessment procedure and is indicated for the treatment of PNH patients. The ECU is able to bind one or two complement fractions 5 (C5), therefore it prevents the cleavage of C5 into C5a and C5b and consequently blocks the formation of the C5b-C9 complex. The clinical effect of blockade of the terminal pathway by the ECU is the abolition of complement mediated intravascular hemolysis. However, the clinical benefits of ECU vary considerably among PNH patients. In 2009, Risitano's group demonstrated that blocking the terminal pathway by the ECU causes an increase in the complement 3 fraction that binds to erythrocytes; this may represent a further disease mechanism in PNH that generates a variable degree of extravascular hemolysis which results in a decrease in clinical benefits by the ECU [Risitano AM et al., 2009].

1.3. The Complement System

The complement system is a group of about 30 circulating and membrane proteins found in all body fluids. It is a complex system characterized by an extremely powerful and therefore finely regulated "cascade" activation mechanism. The proteins that compose it act by interacting with each other and with cell membranes to recognize, opsonize and kill invasive microorganisms, especially bacteria, but also some viruses and parasites. Generally the action of the complementary system is divided into three phases: an initial phase, during which some complement proteins bind directly to surface components of the microorganism or to an antibody bound to its surface; a second phase of amplification, in which the initial components activate subsequent components with an increase, at each step, in the number of molecules involved, and a final stage in which the assembly of a complex of proteins endowed with cytolytic activity, called membrane attachment complex (MAC), which generates holes on the membrane of target cells. The activation of the complement on the surface of the pathogens can occur through three ways [Figure 1].

1) The classical pathway, which represents one of the most important mechanisms of humoral immunity, is activated by the binding of the first component of the cascade (C1q) to the Fc fragment of an antibody bound to its antigen. This binding occurs significantly only with IgM or IgG and determines the activation of two proteases, C1r and C1s, which together with C1q cleave and activate the C4 and C2 proteins. The active domains of these proteins then form a complex called C4b2a, which represents the C3

convertase of the classical pathway. The latter activates C3 to form C3b, a key component in the recognition of pathogens.

2) The lectin pathway is triggered by the activation of a collectin called "mannose-binding lectin" (MBL), which recognizes mannose present on a wide range of microorganisms. The binding of MBL to the mannose of the bacterial wall, or of other microorganisms, triggers the proteolytic enzymatic cascade that leads to the activation of C4 and C2 and, therefore, the formation of C3 convertase.

3) The alternative pathway is independent of binding to an immune complex. Under normal conditions, it is constantly activated at a very slow rate (tickover) by the continuous splitting of the circulating C3 with the formation of very small quantities of C3b. This if it remains in circulation, it is quickly inactivated; if instead it binds to the surfaces of pathogens it binds to a plasma protein called factor B. After binding to C3b (C3bB), factor B loses a small fragment (Ba fragment) by means of a protease called factor D. The fragment residue, Bb, remains bound to C3b constituting the C3b-Bb complex which represents the C3 convertase of the alternative pathway. The C3 convertases, produced through the three pathways, split and activate large quantities of C3 and lead to the formation of C5 convertase (C3bBbC3b and C4b2a3b) by association of some C3b molecules with the same C3 convertases. The cleavage of C5 by the C5-convertases initiates the formation of the lytic complex (MAC) by association of the C5b-C9 proteins, which will lead to the elimination of the target cell. The complement system is therefore important for cellular integrity, tissue homeostasis and activation of the adaptive immune response. The activated complement mediates the removal of microorganisms and facilitates the elimination of dead or modified

cells, removing apoptotic bodies and cell debris. The alternative route also constitutes a spontaneous and constant immuno-surveillance system. The functional multiplicity of this complex system therefore requires a very fine adjustment that allows the discrimination of the self from the non-self, that is, the discrimination of the host cells from the infectious agents. This regulation is carried out by numerous molecules, the complement regulators, which operate at all levels and are classified into three main classes: regulators in the fluid phase, regulators bound to the surface of host cells and membrane receptors responsible for the clearance of complementary fractions [Figure 2]. Some of these receptors also have additional activities: they are involved in the interaction of cells with the extracellular matrix and in the interaction of the complement cascade with other systems such as the coagulation cascade. Fluid phase regulators are distributed in human plasma and body fluids such as synovial and vitreous fluids. The main regulators of the alternative pathway are factor H and the FHL1 protein, which act as cofactors of factor I and participate in the degradation of the C3 convertase of the alternative pathway; the properdin which stabilizes the convertases of the alternative pathway and the carboxylpeptidase N which inactivates the anaphylactic peptides C3a and C5a. Soluble regulators of the classical pathway and of lectins include the C1 inhibitor (C1INH) which blocks serine proteases and inactivates C1r and C1s and the C4-binding protein (C4BP) which blocks C4 and therefore C3 convertase classic way. The host cell membrane is equipped with numerous complement regulators, including CR1 and CR2 proteins, C3 receptors and its metabolites, involved in the removal of immune complexes, in the activation of phagocytosis and in the regulation of C3 cleavage; CD55 (also known as DAF), a protein that contributes to the

degradation of C3 convertase and CD59, a protein that inhibits the formation and assembly of the complement terminal complex (MAC).

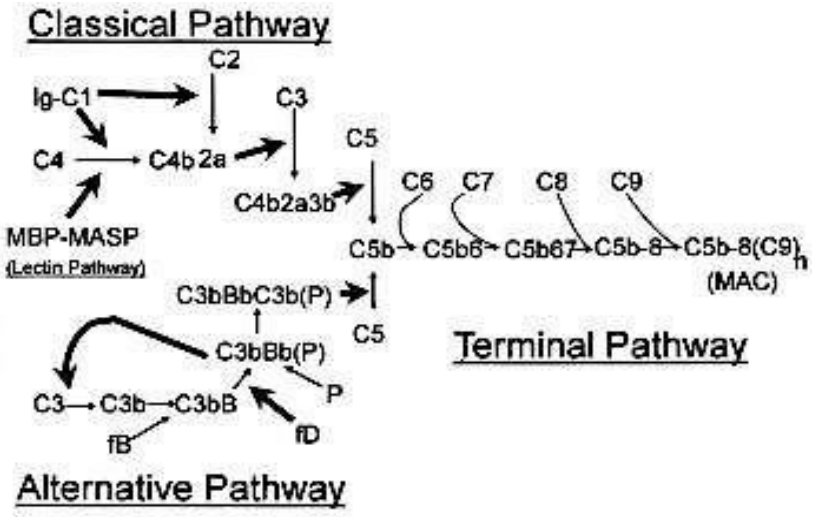


Figure 1. Constitutive pathways of the CS. **Morgan, 2000:** **Complement Methods and Protocols. Humana Press.**

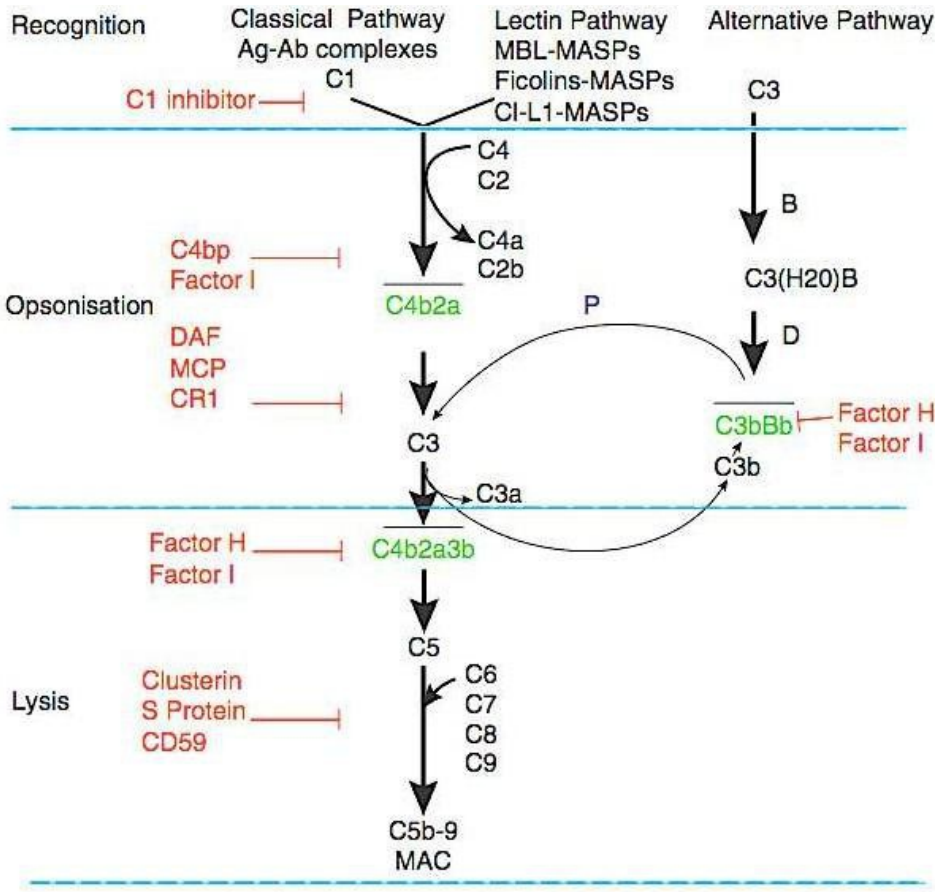


Figure 2. The diagram illustrates in green the convertases and in red the regulators of the classical and alternative pathways of the CS. Michela G., 2013: The CS: Methods and Protocol. Humana Press.

1.4. Epidemiology

The most relevant source from which the data of PNH patients from all over the world are drawn is represented by the International PNH Registry which was established in 2003.

The criteria for patient inclusion include:

- clinical diagnosis of PNH;

- PNH clone ≥0.01% of all blood cells;

- no age limit.

The incidence of PNH is 1-1.5 cases per million, slightly higher in some regions [Socie G. et al., 2016; Yu F. et al., 2016].
The disease is more frequent in Asia [Munoz-Linares C. et al., 2014] and in 30–50 years aged population.
The registry data shows that PNH is rare in children and starts to be clinically evident in adolescence [Ware R. E. et al., 1991; Naithani R. et al., 2008; Curran K. J. et al., 2012; van den Heuvel-Eibrinik M. M. et al., 2005].
PNH has a female predominance (55%) and the specific clinical manifestations may vary in different ethnicities [Nishimura J. et al., 2004].
Thrombotic events are more frequent in the Western compared to Asia [Hill A. et al., 2013].
Median survival of PNH patients was 10 years in the 1990s [Socie G. et al., 1996; de Latour R. P. et al., 2008], but after the introduction of ECU in 2007, PNH patients can live relatively normal lives [Kelly R. J. et al., 2011] except those in which aplastic anemia is associated who have a less favorable prognosis.

The registry data show about 80% of PNH patients are on ECU and that PNH patients die more frequently of bone marrow failure.

1.5. Etiophatogenesis

PNH is a clonal disease due to a somatic mutation of PIGA gene in the hematopoietic stem cell. In 1970 Oni et al. showed the first scientific evidence supporting the hypothesis of monoclonal genesis [Oni S.B. et al., 1970], when a 26-year-old Nigerian woman was diagnosed with PNH and subsequently died of amoebic colitis. The patient's RBCs showed mosaicism with respect to glucose 6-phosphate dehydrogenase, as some RBC had variant A and some RBC had variant B of this enzyme. RBCs carrying the PNH anomaly had only variant B, suggesting that they all belonged to a single abnormal clone [Figures 3, 4].

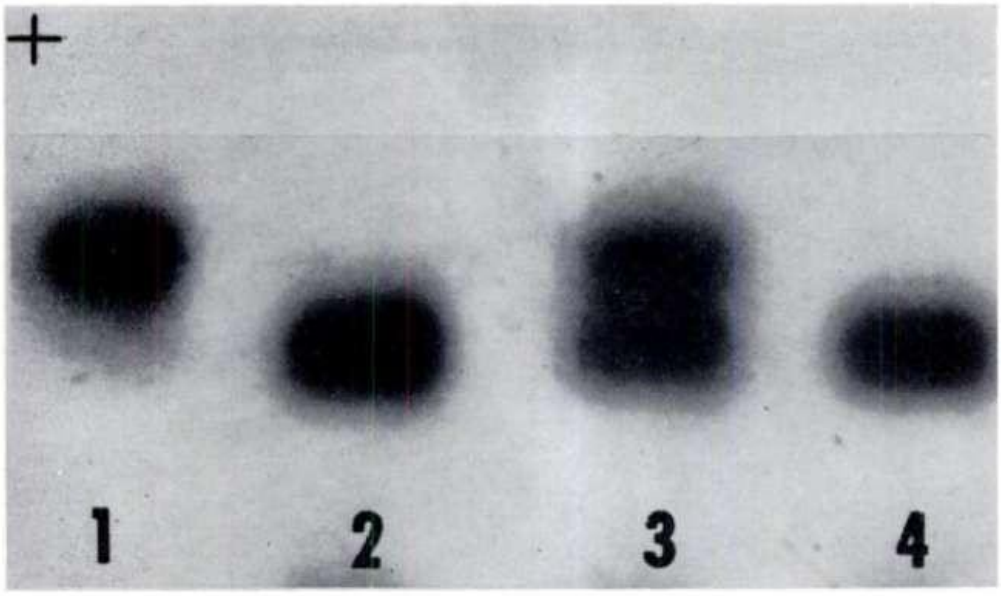


Figure 3. Analysis of glucose 6-phosphate dehydrogenase from patient whole hemolysate and from PNH abnormal cells. (1) Variant A, control; (2) Variant B; (3) Whole hemolysate; (4) PNH cells [Oni SB et al., Paroxysmal nocturnal hemoglobinuria: evidence for monoclonal origin of abnormal red cells. Blood, 1970].

Patients with PNH have clonal blood cells and don’t express various glycophosphatylinositol (GPI) anchored proteins on their surface such as: the monocyte differentiation antigen CD14 (CD14), the immunoglobulin III-B (CD16b) gamma-region receptor Fc, the CD48 antigen (CD48), CD55 and CD59. The study of B-lymphoblastoid and T-lymphoblastoid cell lines showed that GPI biosynthesis was impaired in these cells in PNH patients [Armstrong C, 1992; Hillmen, 1993; Hikada, 1993]. Normally GPI is synthesized starting from phosphatidylinositol thanks to 11 reactions that happen in the endoplasmic reticulum by adding monosaccharide molecules [Kinoshita, 2014; Kinoshita, 2016]. Then, GPI-anchored proteins are transported from the endoplasmic reticulum and Golgi apparatus to the cell surface [Kinoshita, 2016]. In PNH, the first reaction of GPI biosyntesis in defective [Armstrong C, 1992; Takahashi M, 1993; Hillmen, 1993], as consequence, blood cells have defective surface expression of various GPI-anchored proteins. PNH cells lack or have severely reduced activity of phosphatidylinositol [PI]-N-acetylglucosamine (GlcNAc), the PIGA-encoded enzyme that mediates the first step of the GPI biosynthetic pathway. Therefore, the GPI molecules that are competent for attachment to proteins are not generated.

Healthy HSC PNH HSC

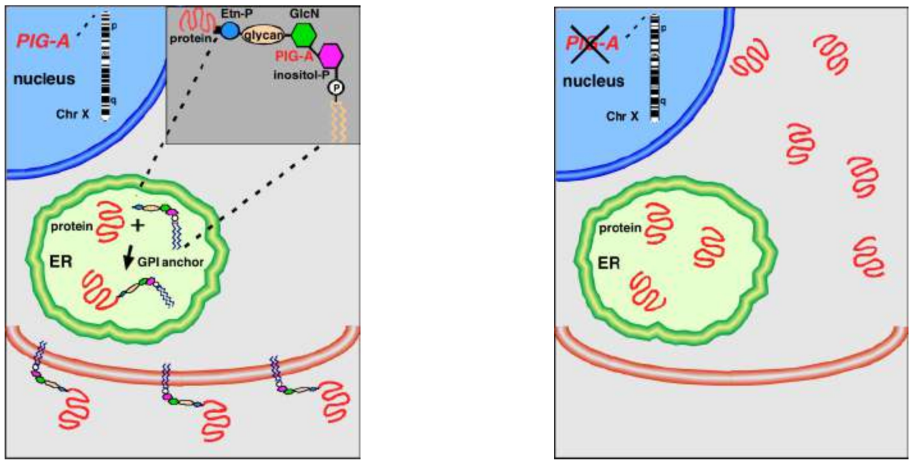


Figure 4. Pathogenesis of PNH. In PNH, mutation of the *PIGA* gene on the X chromosome leads to impaired synthesis of the glycosphatidylinositolyl anchor, which is necessary for numerous molecules to bind to the plasmatic membrane [Luzzatto, 2006].

1.6. Pathophysiology

As a consequence of the clonal expansion of the HSC mutated in the PIGA gene, the generation of populations of mature cells (granulocytes, monocytes, erythrocytes and platelets) without the molecules linked to the GPI anchor occurs. This pathophysiological mechanism, namely the absence of GPI linked molecules, results at a clinical level in intravascular hemolysis and high thrombotic risk. Bone marrow failure of varying degrees, on the other hand, is likely related to aplastic anemia.

1.6.1. Intravascular hemolysis

Normally the complement regulators CD55 and CD59 are on the cell's surfaces and protect host cells from the effects of complement activation. In patients with PNH, a variable fraction of erythrocytes does not have the aforementioned regulators on their surface and this makes them more susceptible to complement attack. Consequently, complement activation following infections and other events causes intravascular lysis of PNH red blood cells and causes paroxysmal hemolysis. Furthermore, since the alternative pathway is always active as an alert mechanism against infections, patients with PNH also constantly present chronic intravascular hemolysis [Figure 5, A, B, C].

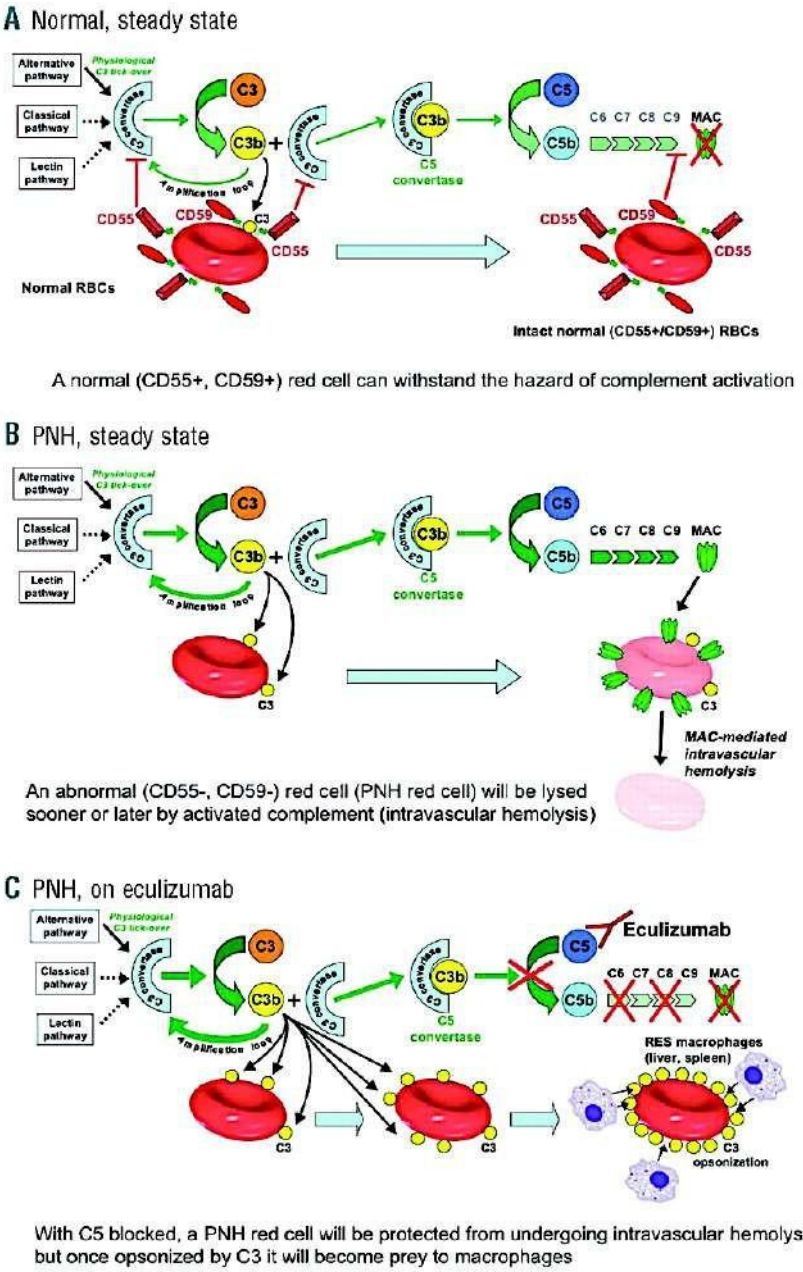


Figure 5: A, B, C. Mechanism of intravascular hemolysis in PNH and block on ECU [Luzzatto et al, 2010].

1.6.2. Thrombosis

PNH is associated with a high risk of thrombotic event. The mechanisms underlying this condition are not yet well understood [Hill A, 2013]. The main contributors that have been proposed are represented by intravascular hemolysis and activation of PNH platelets. The fact that the therapeutic block of complement at the C5 level with ECU reduced the number and severity of thrombotic events suggests that uncontrolled complement activation is involved in this mechanism [Hill A, 2013]. The hypothesis that has been proposed is that the uncontrolled activation of the complement can lead to a non-specific activation, at various levels, of the coagulation cascade and platelets with a consequent increase in thrombotic risk in PNH patients.

Thrombosis is the leading cause of death in PNH patients. They generally involve unusual sites, such as mesenteric, cerebral, or dermal veins; arterial events are less common. Before the advent of anti-complement therapy, about 40% of PNH patients experienced thrombotic events. Currently, the risk in treated patients appears to be similar to age-matched controls. Intra-abdominal thromboses (eg, hepatic, portal, mesenteric veins) account for two-thirds of thrombotic events in PNH patients, followed by intracerebral sites (10-20%) and other sites (eg, skin , lower limbs).

The main risk factor associated with an increased risk of thrombosis in PNH patients is the size of the PNH clone and / or the degree of intravascular hemolysis: in fact, individuals with> 60% of PNH granulocyte clone appear to be at higher risk.

Pathophysiologically, the factors contributing to hypercoagulability in PNH patients include:

- depletion of NO from circulating free hemoglobin;
- procoagulant microparticles released by platelets;
- deficiency of glycosylphosphatidylinositol (GPI) - anchored fibrinolytic and anticoagulant factors;
- increased levels of the complement component, C5, which generates proinflammatory factors and prothrombotic cytokines.

1.6.3. Bone marrow failure

In 1961, Lewis and Dacie were the first to underline the association of PNH with aplastic anemia [Dacie JV, 1961] by describing cases of patients who developed aplastic anemia before PNH and cases of patients who had evidence of hypofunctional bone marrow in the absence of a previous history of PNH. In the following years, always supporting the existence of the relationship between PNH and aplastic anemia, epidemiological studies showed that at least 75% of patients with PNH presented both granulocytopenia and/or thrombocytopenia and, since the survival of these was normal in the blood, this showed that the cause of cytopenia was due to a decrease in production at the medullary level. In addition, several laboratories showed that culture of bone marrow cells from PNH patients, including those with normal functioning marrow, produced few colonies, similar to bone marrow culture from patients with aplastic anemia [Rotoli, 1982]. Furthermore, bone marrow analysis of patients with aplastic anemia revealed that cells lacking GPI-bound proteins were present in abnormal numbers in most patients, considering that a small proportion of GPI negative cells may also be present in the bone marrow. many healthy individuals. From the aforementioned evidence, it is clear that PNH can only arise in the marrow of patients suffering from aplastic anemia and the clinical phenotype can vary from subclinical to severe forms [Figure 6]. Aplastic anemia is due to an immunological attack on hematopoietic stem cells [HSC] and stem cells without GPI anchor may be able to survive despite the attack. Subsequently, there is an increase of clone size in some of these patients that results in controlled growth of the clone due to the acquisition of a second somatic mutation in the GPI-deficient cell line. As a result, the PNH clones expand because they escape the negative selection exerted by self-reactive T cells against normal GPI + hematopoiesis. PNH is expressed in its clinically evident form when the clone increases up to 5-10% of the total bone marrow production. In support of the immunological hypothesis, there is the

presence of cytotoxic T lymphocytes that would produce interferon gamma in response to cells presenting the GPI anchor via the molecule CD1d, a molecule documented in almost all patients with PNH and in three quarters of aplastic patients. [Gargiulo L, 2013; Gargiulo L, 2007]. The aforementioned CD8 + CD57 + cytotoxic T lymphocytes, significantly more abundant in PNH patients than in healthy subjects and enriched in invariant TCRα chain, appear to have homologous TCR-beta sequences in PNH patients [Gargiulo L, 2007] and would therefore be able to recognize and destroy normal cells that synthesize the GPI molecule, sparing PNH cells that do not synthesize it. Therefore, PNH patients actually have two diseases: hypoplasia/aplastic anemia caused by immunological attack of hematopoietic stem cells; PNH caused by the somatic mutation that involves the absence of proteins linked to the GPI anchor.

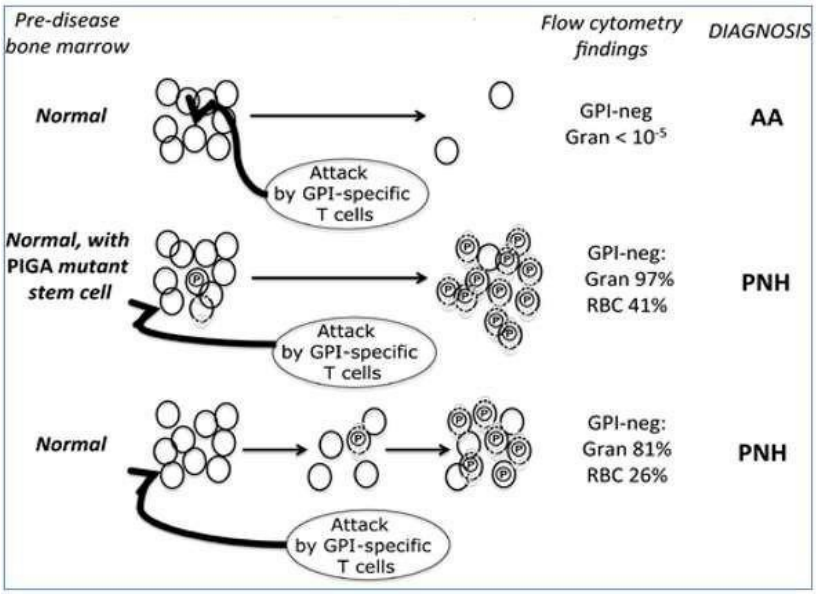


Figure 6. Genesis and flow cytometric profile of PNH phenotypes [Luzzatto, 2020].

1.7. Classification

The International PNH Interest Group (IPIG), is a global professional company dedicated to improving and expanding knowledge about PNH and related disorders in order to improve patient treatment and care. In order to improve the therapeutic management of PNH patients, the IPIG has adopted a classification of patients by dividing them into three categories on the basis of clinical characteristics:

1) Classical PNH (in which patients have clinical manifestations of hemolysis or thrombosis);
2) PNH in the context of other primary bone marrow disorders (such as aplastic anemia or myelodysplastic syndromes);
3) Subclinical PNH, in which patients have low percentages of PNH clones, but no clinical or laboratory evidence of hemolysis or thrombosis [Table 1].

Table 1. Classification of clinical phenotypes in PNH [Parker C, 2005].

Subsequently, the International PNH Registry adopted a classification first used by de Latour et al. in 2008 which includes hemolytic [or classical] PNH, aplastic anemia-PNH and intermediate PNH [Table 2].

Table 2. Classification criteria in PNH. [de Latour RP, 2008]

Patients with hemolytic PNH are generally characterized by:

- neutrophil and platelet counts within the normal range;
- levels of lactate dehydrogenase above double the upper physiological limit (≤1.5x ULN) indicative of intravascular hemolysis;
- normocellular bone marrow;
- increased reticulocyte count;
- PNH granulocyte population> 50%.

Patients with aplastic anemia-PNH (acquired aplastic anemia with a low to moderate percentage of the PNH clone) have:

- severe pancytopenia,
- hypocellular bone marrow with cellularity and / or morphology that meets the criteria for aplastic anemia or MDS;
- relatively low reticulocyte count
- lower percentage of PNH granulocytes.

The intermediate category includes patients with a PNH clone identified by flow cytometry who do not meet the criteria for both of the above categories.

However, it must be emphasized that the category to which a patient belongs may change over time depending on the type of evolution of the disease: for example, patients initially classified as belonging to the aplastic anemia-PNH category may experience an expansion of the PNH clone with improvement. of hematopoiesis, but with the establishment of a hemolytic PNH. More rarely, patients with hemolytic PNH may experience deterioration in bone marrow function and thus develop "aplastic anemia-PNH syndrome".

1.8. Diagnosis

The main clinical manifestations of PNH include hemolysis, thrombosis and bone marrow sufficiency. Although the symptoms are concerned, the patient can report fatigue, lethargy, malaise and asthenia in most cases, while hemoglobinuria is the initial symptom only in about 25% of patients [Parker C, 2005]. Usually from the anamnesis we can deduce history of episodic dysphagia, abdominal pain and male impotence.

As part of the laboratory tests, certainly the presence of non-spherocytic anemia, with signs of intravascular hemolysis, negative to the Coombs test can induce the suspicion of diagnostic PNH. Reticulocytosis reflects the response to hemolysis, although the reticulocyte count may be inadequate to the degree of anemia due to the underlying bone marrow failure; serum concentration of lactate dehydrogenase is always high in patients with clinically significant hemolysis and has a valid index of the extent of intravascular hemolysis. In most patients there is a martial deficiency due to chronic hemoglobinuria and hemosiderinuria, even in the absence of macroscopic hemoglobinuria. Complications of PNH include venous thrombosis, which typically occurs in atypical locations [Budd-Chiari syndrome; mesenteric, dermal or cerebral veins]; arterial thrombosis is a much less common complicance. Different degrees of leukopenia, thrombocytopenia and relative reticulocytopenia can be find in the blood count, which could reflect an underlying bone marrow failure condition.

The clinical diagnosis of PNH should always be confirmed by flow cytometry to determine the absence or severe deficiency of GPI-anchored proteins on at least two or more peripheral blood cell lineages. Bone marrow should not be used outside the research context because the results are often more difficult to interpret due to the changes associated with differentiation in the expression of certain GPI-bound proteins used for assessment in both red blood cell maturation and of white blood cells.

The main clinical indications for requesting a confirmation flow cytometric examination, where there is clinical and / or laboratory evidence suggestive of PNH, are the following:

- Coombs-negative hemolytic anemia;
- aplastic anemia;
- unexplained pancytopenia;
- unexplained thrombosis.

The validity of the flow cytometric diagnosis of PNH is based on compliance with the consensus guidelines published in 2010 [Borowitz M. J., 2010] for detecting GPI-anchored protein deficient blood cells using a combination of FLAER and different monoclonal antibodies.

The proteins anchored via the GPI anchor can be detected after labeling the cells with monoclonal antibodies or a reagent known as fluorescein-labeled proaerolysin (FLAER), which binds to the glycan portion of the GPI anchor. The fluorescent aerolysin or FLAER (Pine-wood Scientific Services, Victoria, BC, Canada) is the reagent used to study the antigens bound to leukocytes through the anchor-GPI. FLAER is a fluorochrome conjugated inactive variant of protein aerolysin that forms bacterial-derived channels, which binds specifically to GPI anchors. FLAER is best used on nucleated cells; it does not stain red blood cells, as they express high levels of glycophorin, a protein that binds to aerolysin and interferes with the assay.

To perform flow cytometric analysis of red blood cells, CD59 is used which allows to recognize and quantify cells that are devoid of expression of GPI-anchored proteins (type III cells), partially deficient (type II cells) if they are present and distinguish them from normal red blood cells (type I cells) [Figure 7].

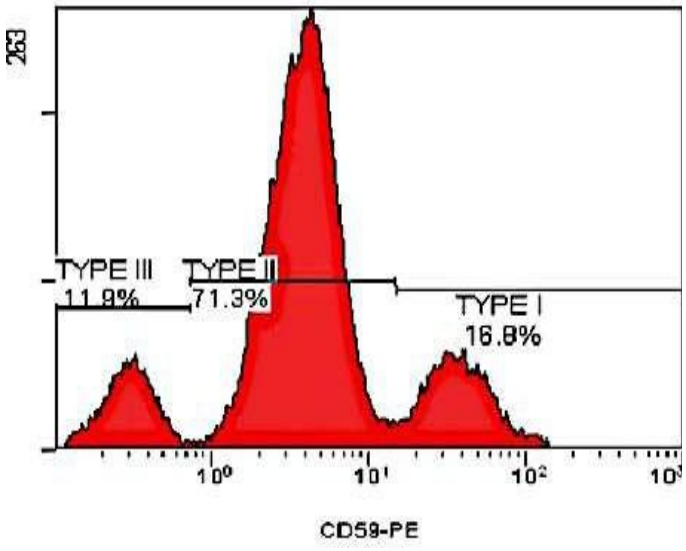


Figure 7. Analysis of CD59 (clone MEM-43 from Invitrogen) expression on RBCs in a patient with PNH showing separation of Types I, II, and III cells. Borowitz M. J., 2010.

Testing of red blood cells alone in a routine test is not adequate for evaluating patients with PNH: in fact, since hemolysis and transfusion can greatly underestimate the size of the PNH clone, it is always necessary to analyze also the white blood cell clone.

To distinguish GPI positive from GPI negative granulocytes, CD16 combined with another reagent is usually used.

CD14 is the GPI-bound marker expressed on monocytes that is commonly used to detect monocyte clones.

Red blood cell staining provides useful information and is significantly better at demonstrating partial antigen deficiency than granulocyte analysis. Although there is no specific cut-off that determines when PNH patients are likely to be symptomatic, patients with> 20% type III red blood cells usually show clinical signs and symptoms associated with intravascular hemolysis. In contrast, patients with large Type II populations in the absence of significant Type III populations may exhibit moderately elevated reticulocytosis and LDH, but less hemolysis than a patient with an equivalent number of Type III cells.

Based on the results of laboratory studies, bone marrow analysis and flow cytometry, patients can be placed in one of three categories according to the recommendation of the International PNH Interest Group [Parker, 2008] [Table 2].

1.9. Management of PNH patient

We can divide the management of the PNH patient into two eras in relation to the development and availability of anticomplement therapy:

1) treatment of the PNH patient before the advent of anti-complement therapy;
2) treatment of the PNH patient in the era of anticomplement therapy.

1.9.1. Era pre-anticomplement therapy

In the era preceding the discovery of anticomplement therapy, the management of patients with PNH for many years was essentially based on two main therapeutic options [Luzzatto, 2011]:
- supportive therapy;
- etiopathogenetic therapy.

1.9.1.1. Supportive therapy

Supportive PNH care can be outlined as follows:
- vitamin support with folic acid in consideration of the high bone marrow turnover in response to hemolysis;
- martial therapy to be introduced in case of iron deficiency caused above all by perpetual hemosiderinuria;
- erythrocyte transfusions in case of symptomatic anemia;

- anticoagulant prophylaxis;
- thrombolysis for complicated cases.

1.9.1.2. Etiopathogenetic therapy

Aetiopathogenetic therapy represents the only treatment capable of modifying the natural history of the disease and, currently, it is reserved only for selected cases in consideration of the serious complications related to it:

- allogeneic HSC transplant;
- immunosuppression with anti-lymphocyte serum, cyclosporine and steroids;
- androgens.

1.9.2. Era of anti-complement therapy

1.9.2.1. ECU: the first monoclonal antibody against C5

The introduction of ECU, a humanized monoclonal antibody against the C5 complement component, has radically changed the management of PNH [Hillmen, 2013].

Blockade of the terminal complement cascade by ECU abrogates intravascular hemolysis, reduces transfusion requirements, and decreases the risk of thrombosis in most patients with hemolytic PNH, and this has been extensively demonstrated in a randomized study, in double-blind, placebo-controlled 26-week trial (C04-001) [Hillmen, 2006] and in the 52-week single-arm study (C04-002) [Brodsky, 2008] and subsequently in a long-term extension study (E05-001) [Hillmen, 2007]. Therefore, the introduction of ECU in the treatment of PNH has radically changed the standard of care for patients with PNH. However, although ECU has been shown to be effective in controlling intravascular hemolysis, the hematological response to ECU is variable: in fact, a considerable percentage of PNH patients (18 to 30% in different series) may remain dependent on transfusions. In addition, even if PNH patients do not require transfusions, they can still have chronic anemia. In this regard, the Italian Network of PNH Centers in 2009 clearly documented that in a series of 50 patients despite almost all lactate dehydrogenase levels being normalized, 81% had achieved transfusion independence, but only 37% had reached hemoglobin levels close to normal (≥ 11 g/dL) [Risitano AM, 2009].

Thus, the hematological response of patients to ECU therapy can be highly heterogeneous and this may be due to various factors which can be classified into three main mechanisms [Table 4]:

- reduced production of red blood cells;
- persistent intravascular hemolysis;
- de novo extravascular hemolysis.

1.9.2.1.1. Reduced production of red blood cells

There are 3 causes of decreased red blood cell production that are commonly present in PNH patients and could affect the hematological response to ECU:

9.2.1.1.1) bone marrow failure: As previously reported, some degree of bone marrow failure is present in every patient with PNH. Therefore, the clinical picture of each individual PNH patient is the result of the relative contribution of bone marrow failure and hemolysis in each individual patient. Therefore, it is important to emphasize that in patients in whom bone marrow failure, up to the form of aplastic anemia (AA/PNH syndrome), is the main cause of anemia, they will not benefit from complement block. On the other hand, in patients in whom bone marrow failure cooperates with hemolysis in determining anemia, the use of complement inhibition is indicated which could help reduce, but not completely abolish the transfusion requirement. Finally, in all cases in which bone marrow failure dominates the clinical picture, further etiological treatments should be considered, depending on the severity of the AA: eg. immunosuppression or allogeneic transplantation of hematopoietic stem cells;

9.2.1.1.2) Vitamin deficiency: in PNH, as in any other hemolytic condition, the increased turnover of red blood cells triggers compensatory erythropoiesis, which may require vitamin support, particularly folic acid. Furthermore, in the absence of complement block, the intravascular hemolysis typical of PNH causes "perpetual hemosiderinuria" leading to iron loss and iron deficiency.

Even in patients treated with ECU, especially at the start of treatment, vitamin support and / or adequate iron supplementation may increase hematological benefit when reduced red blood cell production is mainly due to these deficiencies.

9.2.1.1.3) Low Erythropoietin Levels: Erythropoietin levels are generally elevated in PNH patients and treatment with erythropoietin is not beneficial. However, after complement blockade in some patients, the endogenous erythropoietin level may not be adequate. Therefore, the use of recombinant erythropoietin can be useful as demonstrated in some carefully selected cases [Hill A, 2007; personal observation].

1.9.2.1.2. Persistent intravascular hemolysis

Complement blockade by ECU controls intravascular hemolysis in nearly all PNH patients. The only exception has been reported in the very rare patients carrying an inherited variant of the C5 gene (c.2654G> A, Arg885His) that prevents binding of ECU to C5 resulting in intrinsic resistance to ECU treatment. This genetic variant is polymorphic in the Far East (3.5% heterozygous in Japan and 0.8% heterozygous in the Han Chinese population), but has not yet been found in Western countries [Nishimura J, 2014]. In all other PNH patients on ECU, persistence (or recurrence) of intravascular hemolysis resulting in hemolytic crisis is rare.

The only cases in which PNH patients experience hemolytic crises despite ongoing treatment with anticomplement drugs are:

1) in a small percentage, shortly before the scheduled infusion of ECU (breakthrough pharmacokinetic hemolysis), possibly due to the fact that ECU concentrations unable to control complement activation are reached more rapidly in these patients due to increased high drug turnover rate. In these cases, shortening

the intervals between doses of ECU or increasing the dose of ECU usually can prevent this hemolysis and the consequent need for transfusion;

2) some PNH patients receiving ECU may experience episodes of intravascular hemolysis following an infectious episode or inflammation. The most likely explanation for these hemolytic crises is that the massive complement activation associated with the two clinical conditions mentioned above produces excess C3 convertase and C5 convertase activity that can overcome ECU blockade on C5 (innovative pharmacodynamic hemolysis). This is in line with the observation that in vitro, even concentrations of ECU significantly higher than those of C5 are unable to completely abolish PNH hemolysis of red blood cells after complement activation by serum acidification [Sica M., 2017]. Thus, strong complement activation prevails over C5 inhibition by ECU, possibly due to the high-density generation of C3 products on the surface of red blood cells. Furthermore, another possible mechanism implicated could be the residual end-pathway activity during treatment with ECU which could have important implications for anti-C5 therapy in general [Harder MJ., 2017]. Acute pharmacodynamic hemolysis is an occasional but not uncommon phenomenon which, although it may reduce hemoglobin levels, rarely requires blood transfusions and usually does not benefit from increasing the dose of ECU [Table 3]:

Table 3. Criteria for the definition of breakthrough pharmacokinetic and pharmacodynamic hemolysis during treatment with ECU [Risitano AM, 2019].

1.9.2.1.3. De novo extravascular hemolysis

Classically, PNH patients are negative on the Coombs direct antiglobulin test (DAT) but, when they start treatment with ECU, the DAT becomes positive in most patients [Risitano AM, 2009; Höchsmann B, 2012; Roth A, 2011]. In particular, in almost all PNH patients treated with ECU, a significant fraction of PNH red blood cells (GPI negative) is opsonized by C3. The percentage of PNH red blood cells opsonized with C3 fragments increases progressively from the start of treatment with ECU and usually reaches a relatively stable plateau after 10-12 weeks [Risitano AM, 2009; Sica M, 2017]. In the absence of ECU, the activation of C3 on the surface is followed by the activation of C5 and then invariably by the lysis of the PNH RBCs. Conversely, when C5 is blocked by ECU, C3-coated PNH red blood cells become apparent as they are no longer lysed through the terminal phase of the complement cascade. Eventually, PNH red blood cells, once covered with C3 fragments, can be removed from macrophages, possibly through interaction with complement receptor 3 [Lin Z, 2015], resulting in varying degrees of extravascular hemolysis [Risitano AM, 2009]. The pathogenetic link between opsonization with C3 and extravascular hemolysis is confirmed by the reduced half-life in vivo of 51Cr-labeled red blood

cells with an excess of uptake at the splenic and hepatic level, as well as by the correlation between the extent of the C3 binding with the reticulocyte count [Risitano AM, 2009]. This C3-mediated extravascular hemolysis can limit the efficacy of ECU: it becomes clinically relevant in 25-50% of patients who, in a not small part (25-35%), remain dependent on transfusions [Luzzatto L, 2010; Kelly RJ, 2011; DeZern, 2013]. The size of the C3-bound red blood cell fraction appears to be related to the need for blood transfusions [Risitano AM, 2009].

Binding of C3 to PNH red blood cells and subsequent extravascular hemolysis is a new and unique phenomenon that is related to ECU therapy. However, as will be analyzed in the present work, this mechanism is not specific to ECU, but concerns any treatment based on the C5 block since the control of C5 does not affect the upstream activation of C3 which, therefore, will proceed without obstacles. C3-mediated extravascular hemolysis is present in almost all PNH patients treated with ECU, but becomes a clinical problem for that fraction of patients in which it causes symptomatic anemia or transfusion dependence [Lin, 2015; Notaro, 2018].

Table 4. Causes of inadequate hematological response to ECU and possible therapeutic interventions [Risitano AM, 2019].

1.9.2.2. New complement inhibitors

The knowledge on complement inhibition in PNH, accumulated in recent years both in the clinical and experimental fields, has led to the development of numerous molecules with anticomplement activity that could be useful to improve the treatment of patients with PNH and, possibly, also to overcome unmet clinical needs, such as the problem of extravascular hemolysis.
These new agents with anti-complement action can be divided into two broad categories according to whether the block occurs at the level of the terminal phase or upstream in the proximal phase of the complement activation cascade:

1) terminal complement inhibitors [Table 5];
2) proximal complement inhibitors [Table 6].

In our clinical experience and in the present work, the aspects and characteristics of two new anti-complement drugs have been investigated in particular: ravulizumab (RAVU) and ACH-0144471 (Danicopan).

Table 5. Developing terminal complement phase inhibitors [Risitano AM., 2019].

Table 6. Proximal complement phase inhibitors under clinical development [Risitano AM., 2019].

1.9.2.2.1. Terminal inhibition: RAVU

RAVU is a second generation anti-C5 mAb obtained by reengineering ECU by replacing two amino acids with histidine residues in the complementarity determinant regions (RDF) and two amino acid modifications in the Fc region. The above modifications result respectively in a more efficient pH-dependent dissociation of the antibody from C5 within the lysosomes and increase the affinity of the antibody for FcRn, thus favoring the reuse of the antibody in the vascular compartment and consequently the increased half-plasma life of the drug. The clinical efficacy of RAVU was verified in phase 1/2 studies [Röth A, 2018] and compared with ECU in two phase 3 studies [Lee JW, 2019; Kulasekararaj AG, 2019]. In 2021, the FDA approved the injection of RAVU to treat patients aged one month and older with PNH.

1.9.2.2.2. Proximal inhibition: ACH-0144471

ACH-0144471 (Danicopan) is a factor D inhibitor suitable for oral administration. Complement factor D (CFD) is a specific serine protease that has complement factor B (CFB) as a substrate. By inhibiting CFD, danicopan blocks the formation of C3-convertase and, therefore, prevents the cleavage of C3 which results in the arrest in the activation of the terminal pathway and the lack of formation of MAC. Therefore, danicopan may be effective in both the control of intravascular hemolysis and in the prevention of extravascular hemolysis. The clinical efficacy of ACH-4471 is being studied in two phase 2 clinical trials, in one being studied as monotherapy in naive patients [Risitano et al, 2019], while in another its association with ECU in 11 patients is being studied. with inadequate response to the latter [Kulasekararaj AG, 2019], with administration of 100 to 200 mg three times a day for 24 weeks. ACH-0144471 was associated with an increase in hemoglobin (to 10.3 g / dL at week 24,

compared to 7.9 g / dL at baseline), nearly eliminated transfusions (one unit transfused into one patient, compared to mean of 4.4 units per patient over a 24-week-period) and improved quality of life (mean increase of 11 points in FACIT score). The most common adverse effects were generally mild and included headache, cough, and nasopharyngitis. Currently ACH-0144471 is not yet approved by the FDA or the US EMA.

1.9.3. Infectious risk management in PNH

Both ECU and RAVU increase the risk of infections or sepsis with N. meningitidis, which can rapidly become life-threatening or fatal if not recognized and treated early. Both agents are contraindicated in patients with unresolved Neisseria infection, unless the risks of delaying treatment outweigh the risks of developing a meningococcal infection.

In order to prevent N. meningitidis infections and sepsis, all PNH patients receive meningococcal vaccine (both ACYW135 and serogroup B, either immediately after the first dose of the complement inhibitor or two weeks before the first dose of the complement inhibitor. C5) and antibiotic prophylaxis with penicillin for the duration of therapy with complement inhibitors. This is because invasive meningococcal disease (including non-typable strains that are not included in the vaccine) occurred despite vaccination: in fact, even after vaccination, the risk of Neisseria infection reaches 0.5% per year, i.e., a 1000 times higher risk than the general population [Konar M, 2017; Socié G, 2019].

2. Aims of the study

2.1. Aim 1

Categorize the court of patients who refer to the “Careggi Teaching Hospital” according to the response to treatment with ECU and RAVU and compare the degrees of response among the two different therapies.

2.2. Aim 2

Demonstrate persistence of C3 binding to erythrocytes during the treatment with RAVU.

2.3. Aim 3

Evaluation of the efficacy of proximal complement inhibitor ACH-0144471 in preventing the binding of C3 fragments to PNH erythrocytes.

2.4. Aim 4

Analyze the mechanism of hemolytic crises induced by bacteria.

3. Patients, materials and methods

3.1. Patients

12 PNH patients referring to Careggi Teaching Hospital in Florence entered this study: 7 of them were enrolled in various clinical trials [Lee JW, 2019; Kulasekararaj AG, 2019] and 5 were complement-inhibitor naive. As part of our analysis, we included also 7 patients referring to the Hospital of Naples. Peripheral blood samples were collected during normal clinical checks and after signing informed consent according to protocols approved by the Ethics Committee.

3.2. Material and methods

3.2.1. Clinical studies

3.2.1.1. Phase 3, randomized, open-label, actively controlled study of ALXN1210 versus ECU in complement inhibitor-naïve adult patients with PNH

The ALXN1210 study (NCT02946463, EudraCT 2016-002025-11; PNH-301) enrolled naïve patients in anticomplement treatments. This was a phase 3, multicenter, randomized, active-controlled, open-label study conducted in 123 centers in 25 countries. After a 4-week screening period, patients are randomized into two arms for a period of 26 weeks to evaluate the efficacy and safety of RAVU compared to ECU. Afterwards, all patients received RAVU for an extension period of 2 years. RAVU achieved the goal of not inferiority compared to ECU on both end points covering the sea, namely both in terms of the percentage of patients with normalization of lactate dehydrogenase levels and in terms of the percentage of patients who achieved transfusion independence and point estimates for both end points. Headgear have favored RAVU therapy. RAVU therapy was also found to be not less than ECU on the 4 key secondary endpoints, namely, percentage of participants with breakthrough hemolysis, percentage change from baseline in lactate dehydrogenase levels, change from baseline in quality of life according to "(FACIT) – Fatigue score" and percentage of patients with stabilized hemoglobin levels, with all estimates point in favor of RAVU. Thus, having met all the efficacy endpoints both for seas and secondary, with a similar safety profile, the ALXN1210-301 study therefore showed that RAVU administered every 8 weeks is not inferior in efficacy to ECU administered every 2 weeks in patients with PNH naive to C5 inhibitors.

3.2.1.2. Phase 3, randomized, open-label, actively controlled study of ALXN1210 versus ECU in PNH patients treated with ECU

Study ALXN1210 (NCT03056040 EudraCT 2016-002026-36; PNH-302) consisted of a multicentre, randomized, open-label, active control study conducted in 49 centers in 11 countries of 195 PNH patients who were clinically stable on one dose ECU standard. The study consisted of a 4-week screening period followed by a 26-week randomized treatment period and an extension period during which all patients received RAVU for up to 2 years. After stratification based on transfusion history and patients were randomly assigned (1: 1) to 26 weeks of open-label treatment to RAVU or ECU. At the end of the 26-week treatment period, patients treated with RAVU continued the maintenance dose of RAVU every 8 weeks, while patients treated with ECU switched to open-label RAVU for the extension period and initially received a loading dose of RAVU, followed 2 weeks later by maintenance doses always every 8 weeks.

The results of the study demonstrated that RAVU is non-inferior to ECU for the primary endpoint of the percentage change in lactate dehydrogenase and also for the 4 key secondary endpoints: percentage of participants with breakthrough hemolysis, percentage change from baseline of lactate dehydrogenase levels, change from baseline in quality of life according to "(FACIT) - Fatigue score".

In conclusion, this study has shown that PNH patients can safely and effectively switch from ECU to RAVU without changes in efficacy, safety and quality of life compared to ECU. RAVU was also shown to be no less than ECU for all efficacy endpoints, and the complete and prolonged inhibition of C5 determined by RAVU explains the consistent results across the endpoints.

3.2.1.3. Phase 2 open-label study of ACH-0144471 in PNH patients who have an inadequate response to ECU monotherapy

The dose-finding study on ACH-0144471 (NCT03472885 EudraCT 2016-003526-16) aimed to demonstrate that danicopan is a potential treatment for PNH patients with an inadequate response to the ECU and transfusion dependent; The rationale for the study is that factor D blockade can prevent C3-mediated extravascular hemolysis which is believed to be a key clinical component affecting the hematological response to ECU. According to the study design, therefore, in addition to treatment with ECU, patients received additional danicopan initially 100-150 mg TID, with dose increase up to 200 mg TID, based on clinical and biochemical response, at times defined by the protocol. The primary endpoint was the change in Hb at 24 weeks of treatment. Secondary efficacy parameters included: transfusion requirement, effect on lactate dehydrogenase and an exploratory endpoint assessed by "(FACIT) - Fatigue score". After the 24th week of treatment, patients entered the long-term extension phase.

The results of the study demonstrated an improvement in Hb values and asthenic symptoms assessed according to "(FACIT) - Fatigue score" and a drastic reduction in transfusion requirements for all patients. In addition, there was a significant reduction in the binding of C3 fragments and in the absolute reticulocyte count, and in some patients a normalization of total bilirubin, direct and lactate dehydrogenase levels was also achieved.

Furthermore, danicopan was well tolerated and in no case was the treatment discontinued.

In conclusion, the study demonstrated that the addition of danicopan results in clear improvement and clinical benefit for patients with inadequate response to ECU therapy.

3.2.2. Flow cytometry

Analysis of PNH cell populations was performed on peripheral blood samples obtained from patients after signing informed consent. For erythrocyte analysis, 10 µl of whole blood was diluted in 3 mL of PBS.

3.2.2.1. Analysis of PNH erythrocytes

For the study of PNH erythrocytes, 50 µl of the diluted blood was transferred to a cytofluorimetry test tube and 5 µl of a 1:15 dilution of an anti-CD59 monoclonal antibody conjugated with APC (MEM-43 clone, Bio-Rad), was added. Subsequently, the erythrocytes were incubated for 20 minutes in the dark with the antibody. At the end of the incubation the cells were resuspended in ~300 µL of PBS and analyzed with the cytofluorimeter (CytoFLEX S, Beckman Coulter), the results were then analyzed with the CytExpert software (Beckman Coulter).

3.2.2.2. Analysis of C3 binding

For the study of C3 fragments deposited on erythrocytes, 50 µl of diluted blood were transferred to a cytofluorimetric test tube, to which 5 µl of a 1:10 dilution of unmarked anti-C3d NEO (Quidel) primary mouse antibody was added and incubated for 30 minutes with the antibody. At the end of the incubation the cells were washed with PBS and centrifuged for 5 minutes at 300 x g, the supernatant was discarded and 5 µl of a 1:20 dilution of secondary anti-murine anti-immunoglobulin antibody conjugated with PE (Dako) was added to the pellets. After an incubation of 30 minutes in the dark, the erythrocytes were washed with PBS and centrifuged for 5 minutes at 300 x g and the supernatant was discarded. Then, 5 µl of mouse serum was added to block any nonspecific binding sites, and the cells

were incubated for 15 minutes in the dark. At the end of incubation, 5 µl of anti-CD59 antibody conjugated with APC were added and the RBCs were incubated for 20 minutes in the dark with the antibody. At the end of incubation, the cells were resuspended in ~200 µl of PBS and analysed directly at the cytofluorimeter.

3.2.3. Molecular diagnosis of β-thalassemia

DNA was extracted from peripheral blood using a standard methodology using the QIAmp DNA Blood Midi Kit (Qiagen). For the identification of the thalassemic variant β [0] 39, a 447 bp genomic fragment including the I and II exons of the β-globin gene was amplified by polymerase chain reaction [PCR] using the following primers:
Sense primer: 5'-TTGCTTCTGACACAACTGTG-3';
Antisense primer: 5'-CACTCAGTGTGGCAAAGGTC-3'.
100 ng of genomic DNA were amplified in a reaction mixture containing: a reaction buffer (PCR Buffer II 1x), $MgCl_2$ [1.25 mM], deoxy-ribonucleotides [dNTPs 0.2 mM final], sense and antisense primers [final 300 nM], 1.25 U DNA polymerase (Amplitaq DNA Polymerase 5U/µl) and sterile H_2O up to a volume of 25 µl.
The amplification program used included:
a denaturation cycle at 95°C for 3 minutes; followed by 35 cycles at 95°C for 30 seconds, 30 seconds of annihilation at 58°C and 45 seconds of extension at 72°C; followed by a final cycle of 5 minutes of extension at 72 ° C to complete the elongation of all PCR products.
The amplified DNA was separated by electrophoresis on 1.5% agarose gel and, at the end of the electrophoretic stroke, displayed via UV thanks to the use of a non-toxic intercalant (EuroSafe, Euroclone).
Finally, the 447 bp DNA fragment obtained by PCR was subjected to Sanger sequencing (Macrogen) and the results were analyzed with the Codon Aligner software (CodonCode Corporation).

3.2.4. Typing of human blood and heat inactivation of human sera

We collected human serum by standard centrifugation of the whole blood obtained by direct puncture to the right cephalic vein of 20 healthy lab members. We typed whole blood for the AB0 group by standards methods. Then, we heated serum at 56°C for 30 minutes to get inactivated.

3.2.5. Analysis of bacteria's growth in normal human sera

We inoculated the E. coli strain (JM 109 - Competent Cells obtained by Promega Corporation · 2800 Woods Hollow Road · Madison, WI 53711-5399 USA) in two mixtures: one including 50% of A, B and 0 blood type-matched pooled normal human serum and 50% of Luria Broth and in another one including 50% of heat-inactivated pooled normal human serum and 50% of Luria Broth. Then, we incubated overnight the bacterial inoculum at 37°C in a shaking incubator. Then, we evaluate the bacteria growth by spectrophotometric method and measuring the absorbance at a wavelength of 600 nm (OD600) [Figure 8].

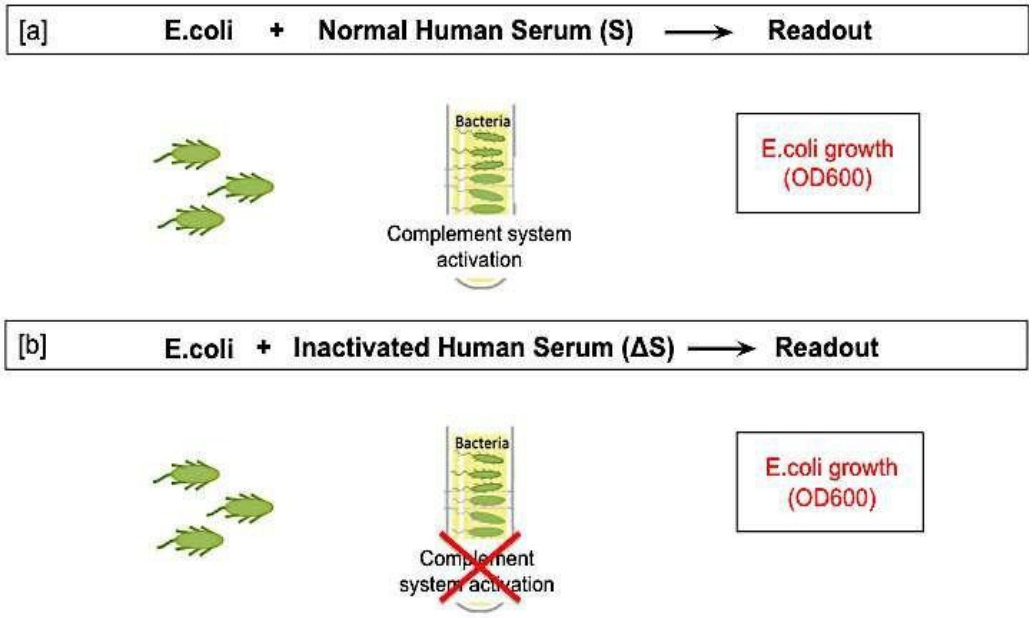


Figure 8. Experiment design for the analysis of bacteria's growth in normal human sera.

3.2.6. Assessment of bystander lysis

We set up E. coli inoculum (JM 109 - Competent Cells Offered by Promega Corporation · 2800 Woods Hollow Road · Madison, WI 53711-5399 USA) in a mixture with 50% AB0 blood type-matched pooled normal human serum [NHS] and 50% of LB as well as with 50% heat-inactivated AB0 blood type-matched pooled normal human serum (Delta) and LB. Then, we added PNH RBCs 10 microL after washing three times in saline solution and keep overnight at 37°C in a shaking incubator. Then, we measured the absorbance by spectrophotometry at 380 nm, 415 nm and 450 nm by using Harboe assay (Harboe M, 1959) [Figure 9].

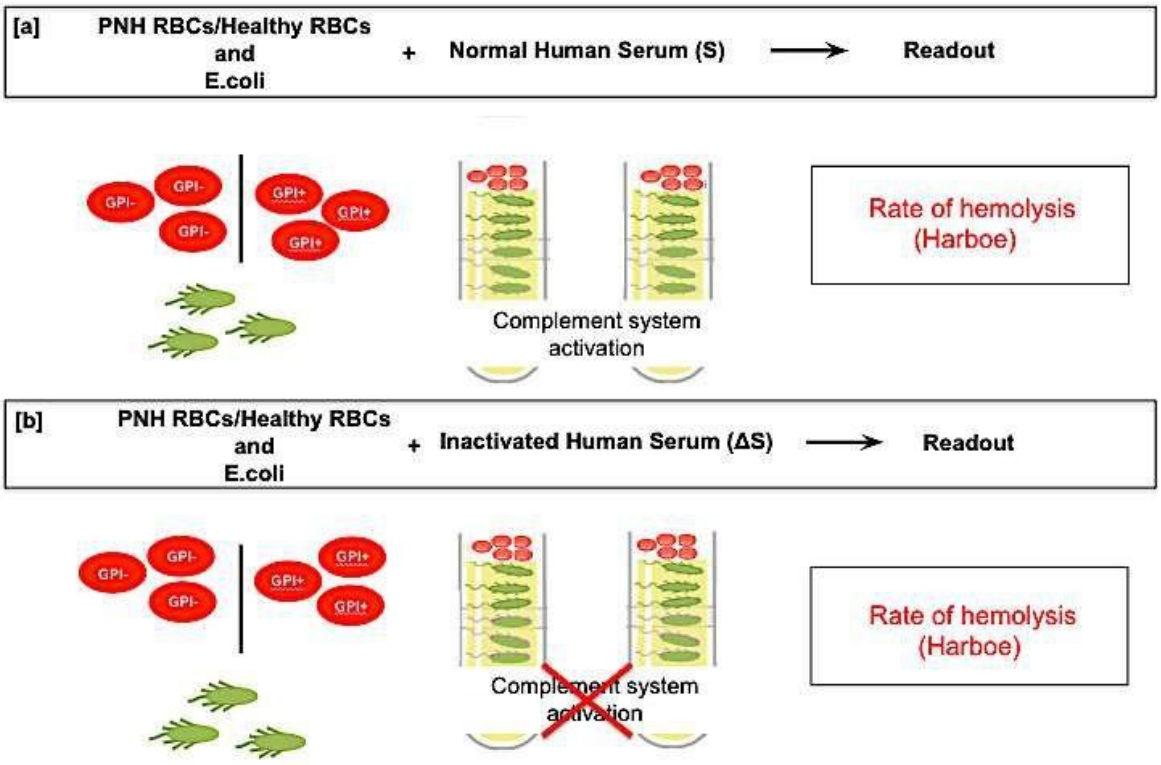


Figure 9. Experimental design for the assessment of potential bystander lysis.

3.2.7. Statistical analysis

The results are expressed as mean ± standard deviation. Statistical analysis was performed by Student t-test for paired two-tailed samples using GraphPad Prism v.5.0 software (GraphPad Software, La Jolla, CA, USA). Significance was accepted for P values <0.05.

4. Results and discussion

4.1. Evaluation and categorizing the hematological response to the treatment with C5 inhibitors

In 2017, two phase three clinical trials were activated at our institution for PNH patients aimed at verifying whether a new anti-C5 monoclonal antibody of the recycling and long-acting type, ALXN1210 then renamed RAVU, dosed every 8 weeks was no less than the standard therapy with ECU dosed every 2 weeks.
A total of 7 patients were enrolled and followed at our Center [Table 7] of which 3 patients naïve to anticomplement therapy (Studio PNH-301) [Figure 9, a] and 4 who were already on ECU therapy (Study PNH-302) [Figure 9, b].

Study	UPN [N=7]	Age	Gender [F/M]	Hb [g/dL]	Reticulocytes [n*109/L]	LDHxULN	RBC CD59-negative [%]
302	26F	35	M	6,6	112.9am	3	40
302	31F	26	M	10,4	191.4am	8	50
302	46F	72	F	17.8	159.5am	5	50
302	50F	30	M	8,3	236.6am	10	60
301	74F	53	F	8,6	184.0am	11.9 am	23
301	84F	75	F	9	5.8 pm	7.8	19
301	90F	74	M	8	142.5am	9.2	47

Table 7. Characteristics of the study cohort [N = 7] before initiation of anticomplement treatment. M = male; F = female; Hb = hemoglobin; LDH = lactate dehydrogenase; ULN = "upper limit of normal".

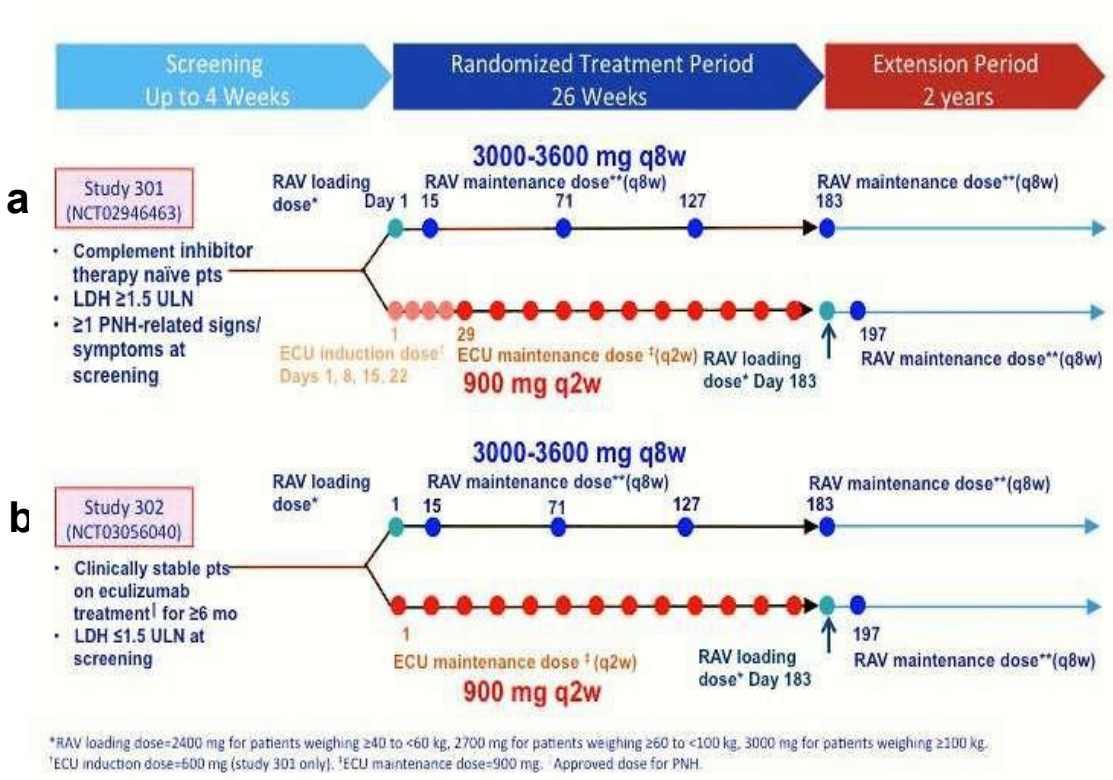


Figure 9. Design of the clinical trials: [a] ALXN1210-301 [Lee JW, 2019] and [b] ALXN1210-302 [Kulasekararaj AG, 2019].

The 3 naïve patients were all randomized to the ECU arm; while, of the 4 patients already on ECU therapy, 2 were randomized to the ECU arm and 2 to the RAVU arm.

Since, therefore, all 7 patients included in our case studies had 6 months of ECU therapy before switching to RAVU, we had the opportunity to compare the hematological response parameters in the same patient before and after the switch from ECU to RAVU and, therefore, to obtain an intra-individual evaluation to be compared with the inter-individual one between patients receiving ECU and patients receiving RAVU, which was also the basis of the two clinical trials.

In these patients we observed that the clinical-laboratory values achieved during the stable phase of ECU therapy did not differ significantly from those obtained during RAVU therapy. In particular, no changes in hemoglobin levels [Figure 10], lactate dehydrogenase levels were observed, reflecting the control of intravascular hemolysis [Figure 11], reticulocytosis which we consider a possible indicator of the presence of extravascular hemolysis [Figure 12].

Figure 10. Trend of hemoglobin [Hb] in Florence's PNH court [N = 7] before [pre-anti-C5] and after anticomplement therapy with ECU [ECU] and RAVU [RAVU]. Blue = 31F; cyan = 84F; purple = 74F; green = 50F; red = 90F; orange = 46F; magenta = 26F; triangular and square indicators express the masculine and feminine genders, respectively. T0 = the indicator expresses the value of Hb detected for each patient immediately before the start of anticomplemental therapy; T1 = the indicator expresses the average of the Hb values measured in the time interval between the beginning and after 6 months of ECU therapy; T2-3-4-5-6-7 = the indicator expresses the value of Hb after 6-12-18-24-30-36 months respectively after the start of RAVU therapy. The above specifications are also to be applied for the subsequent graphs elaborated for the different variables examined: lactate dehydrogenase [Figure 11], reticulocyte count [Figure 12], GPI-negative erythrocytes [Figure 17].

Pair Samples Statistics Hb	Mean	N	Std. Deviation	P value
ECU	99,143	7	173,631	0,516
RAVU	101,857	7	105,424	

Figure 10, table 8. The hemoglobin levels of all PNH patients of Florence's court [N = 7] improve after the start of anticomplement therapy [p= 0,516] and do not undergo significant changes after transition from ECU to RAVU. These specifications are also to be applied to the subsequent ones for the LDH variable [Figures 10], for the reticulocyte count [Figures 11] and for the C3 levels [Figures 15].

Figure 11.

Pair Samples Statistics LDH	Mean	N	Std. Deviation	P value
ECU	1,042	7	0,20702	0,0821
RAVU	1,071	7	0,35456	

Table 9.

Figures 11, table 9. Lactate dehydrogenase [v.n. = 250 U/L] levels in Florence's PNH court [N = 7] improve after initiation of anti-complement therapy [p= 0,001] and do not undergo significant changes in the transition from ECU to RAVU.

Figure 12.

Pair Samples Statistics Abs.Ret. Count	Mean	N	Std. Deviation	P value
ECU	2,924,257	7	0,20702	0,599
RAVU	2,016,300	7	0,35456	

Table 10.

Figures 12, table 10. The reticulocyte absolute count of Florence's court [N = 7] increases after the start of anti-complement therapy [p 0.599] and does not undergo significant changes in the transition from ECU to RAVU.

In 2019, the working group made up of experts from the main PNH Study Centers in America, France, England and Italy, proposed a classification of the hematological response [table 11] of patients on complement inhibitor therapy [Risitano et al., 2019].

This classification has the purpose of defining the unmet clinical needs of the available therapies for each patient. Thus, in this study, patients receiving ECU therapy are classified on the basis of hemoglobin levels, residual hemolysis and transfusion requirements.

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 ≥8 and <8 g/dL, and no response based on hemoglobin <8 g/dL. All hemoglobin, LDH and ARC values should be assessed based on the median value over a period of 6 months.*

Table 11. Classification criteria for the hematological response to anti-complement therapy. [modified from Risitano et al, 2019].

One of the aims of our work was to compare the hematological response to ECU compared to that to RAVU by applying the new classification criteria reported above. For this reason, we evaluated 7 patients at the end of the treatment with ECU and after 36 months of treatment with RAVU. At the end of ECU therapy, two of the three patients enrolled in ALXN 1210-301 achieved a partial response and one patient a

minor response, and three of the four patients enrolled in ALXN 1210-302 achieved a good response and one patient a response. inferior. After switching to RAVU, all patients maintained the same degree of response.

Finally, none of the patients changed the response category from the infusion of at least 2 weeks of ECU to the 8-week infusion of RAVU, confirming the non-inferiority of RAVU with respect to the ECU.

The results of our intra-individual analysis, albeit within the limits of the small number of series, are in line with those obtained from the inter-individual analysis conducted in the two clinical studies [Figure 13].

A. **B.**

Figure 13: RAVU is effective both in the court of patients naïve to anti-complexion therapy [a] [Lee JW, 2019] and in patients previously treated with ECU [b] [Kulasekararaj AG, 2019].

In addition, we applied the above classification criteria to assess hematological response in a cohort of PNH patients treated with ECU evaluated in six international PNH referral centers (Paris, Naples / Avellino, London, Florence, San Paolo and Ribeirão Preto). Hematological response was assessed at 6, 12, and in the last six months of ECU treatment [Figure 14, A]. The analysis found that: 22% of patients met the complete or major response criteria, about 66% were included in one of the two intermediate categories and 12% of patients were classified as minor or non-responder.

Subsequently, our recent work [Debureaux, 2021] has proposed a simplification of the previous classification [table 11] based on only 4 categories instead of 6 [Figure 14 A, B, C]:

- group A: includes patients with complete response and includes patients with complete and main response of the previous classification. The characteristics of this category are independence from transfusion and the absence of anemia - 22%;

- group B: includes patients with good response. The characteristics of this category are independence from transfusion and mild anemia (10-12 g / dL) - 40%;

- group C: includes patients with partial response. The characteristics of this category are persistent anemia (8-10 g / dL) with rare transfusions (≤ 2 units of packed RBC in 6 months) - 26%;

- group D: includes patients with a minor response and includes patients with a minor response and no response from the previous classification. The characteristics of this category are persistent transfusion-dependent anemia - 12%.

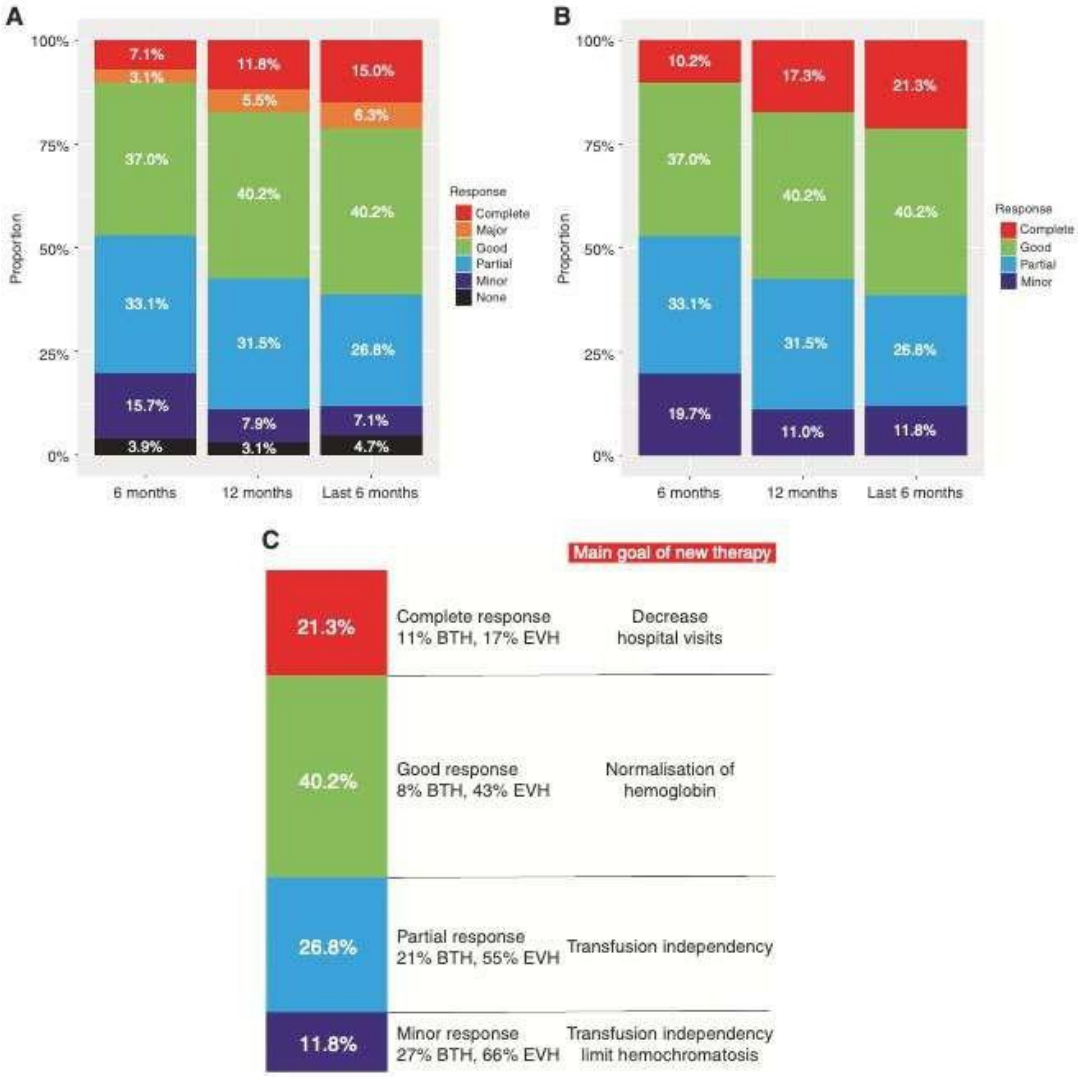


Figure 14. Categorizing hematological response to ECU in PNH: a multicenter real-life study [Debureaux PE, 2021].

According to this simplified classification, 17% of patients showed an improvement in response at the last evaluation, especially in groups C and D. 22% of patients had a reduced response between the initial 12 months and the last evaluation. Patients who did not obtain a hematological response, however, benefited clinically as demonstrated by the reduction in thromboembolism [Risitano AM, 2019]. Only 14% of ECU-treated patients achieved normalization of hemoglobin, ARC and LDH. In this regard, there are several factors that contribute to residual anemia during ECU treatment in our global cohort and include: underlying bone marrow dysfunction (2%), residual chronic intravascular hemolysis (13%), hemolysis acute breakthrough (17%) and C3-mediated extravascular hemolysis (48%) [Risitano AM, 2009] and the proposed classification is decisive for understanding these mechanisms involved in the clinical needs of each patient and, consequently, individualizing decisions therapeutic especially in cases of poor response to ECU [Risitano AM, 2019]. In conclusion, this classification represents a valuable tool for all patients treated with ECU and potentially also RAVU, especially for: the evaluation of the hematological response to one year of treatment with ECU, the design of clinical studies, the identification of target populations, the definition of significant endpoints and the comparison of the clinical results of the new anti- complement agents for PNH.

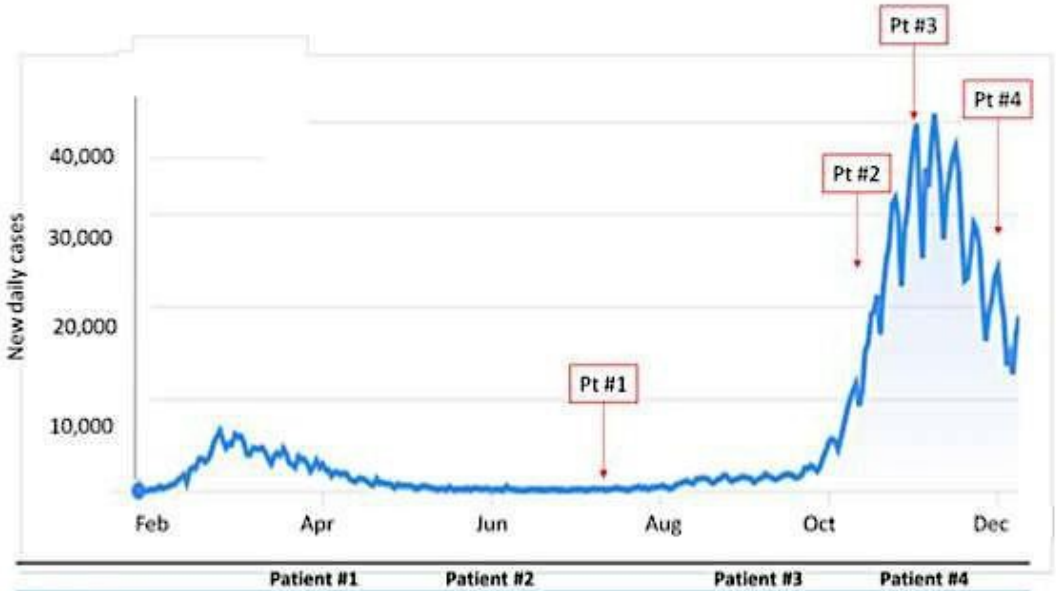
	Complete [n=27]	Good [n=51]	Partial [n=34]	Minor [n=15]	p
AA-PNH at Ecu initiation	10 [37%]	13 [26%]	15 [44%]	10 [67%]	0.05
LDH [median, ULN, IQR]	1 [0.8-1.3]	1 [0.9-1.2]	1 [0.9-1.3]	1.2 [0.9-1.7]	0.84
ECU [1200 mg or < 12 days]	2 [7%]	5 [10%]	6 [17.6%]	3 [20.0%]	0.04
Breakthrough hemolysis	3 [11%]	4 [8%]	7 [21%]	4 [27%]	0.14
Thromboses after Ecu initiation	0	2 [4%]	1 [2%]	1 [5%]	0.75
SAA evolution	0	0	1 [2%]	2 [10%]	0.04
Hemoglobin [g/dL; median, IQR]	13.1 [12.6-13.5]	10.9 [10.3-11.3]	9.2 [9-9.5]	9.5 [8.7-9.9]	< 0.01
Transfusion dependent	0	0	10 [29%]	14 [93%]	< 0.01
ARC [109/L; median, IQR]	111 [71-153]	166 [108-214]	199 [141-261]	220 [155-353]	< 0.01

Table 12. Categorizing hematological response to ECU in PNH: a multicenter real-life study [Debureaux PE, 2021].

4.2. An Italian multicentre survey on covid-19 in patients with PNH

Among the factors inducing hemolytic crises in PNH patients, SARS-CoV-2 infection was also reported [Schüller H, 2021] and in this regard, in 2021, a study conducted among eight Italian reference centers analyzed the occurrence and clinical features of SARS-CoV-2 infection in 156 PNH patients during the pandemic period [Barcellini W, 2021]. The patient cohort suffered from hemolytic PNH with and without concomitant aplastic anemia (AA) or myelodysplastic syndrome and one part had subclinical disease. Almost all of the patients studied were on stable treatment with complement inhibitors at the time of the COVID-19 outbreak and only a small percentage had significant comorbidities. During the observation period, the patients were subjected to at least one nasopharyngeal swab and four were positive, two of which were asymptomatic and two with symptoms [figure 15]; the latter presented a paucisymptomatic clinical picture not requiring hospitalization. It is specified that three out of four patients were being treated with complement inhibitors and one experienced breakthrough hemolysis (BTH). No thrombotic complications emerged from the analysis. In the cohort of patients remaining without SARS-CoV-2 proven infection: eight experienced a febrile episode, two hemolytic crises reported in untreated patients, two patients with a BTH event, and four patients requiring transfusion support. This is the first survey of a large cohort of PNH patients to suggest that, with or without complement inhibition, PNH patients are not at significantly higher risk of SARS-CoV-2 infection than the general population. Furthermore, in support of the above, the results of this study are in line with previous reports in the literature, consisting of case reports or small series of cases, which report a mild course of COVID-19 in patients with PNH, especially if in treatment with complement inhibitors with the exception of a single fatal case of a PNH patient associated with AA [Kulasekararaj AG, 2020; Pike A, 2020]. Given that immune activation and inflammation play an important role in the clinical severity of

COVID-19 pneumonia, given the critical and damaging role of complement activation previously demonstrated in experimental models of SARS-CoV pneumonia and Middle Eastern respiratory syndrome (MERS) -CoV, as well as in SARS-CoV-2 infection in humans [Risitano AM, 2020] and given that complement inhibition appears to be beneficial in both in vitro models of SARS-CoV-2 infection and in vivo COVID-19 [Yu J, 2020; de Latour RP, 2020], it is concluded that complement inhibition did not increase the SARSCoV-2 infectious risk in PNH and could mitigate the clinical severity of COVID-19.



	Patient #1	Patient #2	Patient #3	Patient #4
Age/ sex	55/F	58/F	41/F	39/M
Demographics	Caucasian	Caucasian	Caucasian	Caucasian
PNH type	Haemolytic	Haemolytic	Haemolytic	Haemolytic
Comorbidity	Breast cancer	DVT, arterial hypertension	None	None
Prophylactic anti-coagulation/type	No	Yes/warfarin	No	No
PNH clone % (G/M/E)	68/69/10	87/80/38	75/83/21	NA
Anti-complement therapy	No	Yes, eculizumab 900 mg i.v. q14d since 2008, then ravulizumab 490 mg s.c. weekly since January 2020)	Yes, ravulizumab 3000 mg i.v. q8w	Yes, eculizumab 900 mg i.v. q14d
Baseline LDH (x ULN)	5	1.1	1.3	NA
Symptoms	Fever, ageusia	None	None	Fever
SARS-CoV-2 NP swab	Positive	Positive	Positive	Positive
Hospitalisation	No	No	No	No
COVID-19 treatment	Prophylactic LMWH, azythromycin	None	None	None
LDH (x ULN)	3.9	1.2	2.8	NA
Oxygen requirement	No	No	No	No
BTH	No	No	Yes	No
Thrombotic complications	No	No	No	No
Sequelae	Resolved	Resolved	Resolved	Resolved

Figure 15. COVID-19 in patients with paroxysmal nocturnal hemoglobinuria: an Italian multicentre survey [Barcellini W, 2021].

4.3. Binding of C3 fragments to PNH erythrocytes during C5 inhibition: persistence during treatment with RAVU

To demonstrate the hypothesis that the phenomenon of C3 binding is not exclusive to ECU therapy, but that it is linked more generally to the blockade of C5 we have detected with cytofluorimetric method.

The presence of C3d fragments in the same patient during the stability phase during ECU therapy and after initiation of RAVU therapy. Cytofluorimetric analysis has shown that C3d is deposited on RBCs, spared from intravascular hemolysis thanks to C5 blockade, both during therapy with ECU and during therapy with RAVU [Figure 16, A, B, C]. Specifically, in the 7 patients enrolled in the trials we observed that the level of C3d binding during ECU treatment was not significantly different from that observed during RAVU treatment in intraindividual and interindividual comparison. The C3d binding analysis was extended to 6 patients from other Italian centers for which we were able to compare the level of C3d binding after at least 6 months of treatment with ECU [median 6 months; range: 6-12 months] with the level of C3d binding after 36 months of RAVU in a total of 13 patients. Analysis of these 13 patients confirmed that the use of two different C5 inhibitors did not result in significant changes in C3d binding [Figures 17-18, table 13-14].

3.A. B. C.

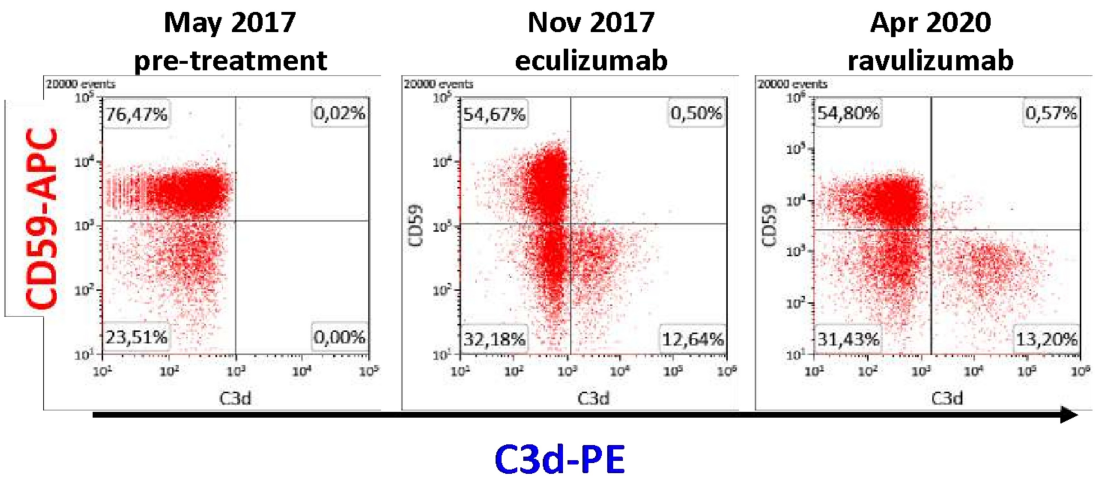


Figure 16. Flow-cytometric detection of PNH RBCs C3d+ before anti-C5 inhibition [A], on ECU [B] and on RAVU [C].

Figure 17. GPI-negative erythrocyte clone trend PNH RBC [%].

Pair Samples Statistics CD59- [Florence and Naples]	Mean	N	Std. Deviation	P value
ECU	47,410	13	56,380	0,0622
RAVU (≥36 months)	30,630	13	33,200	

Table 13.

Figure 18. Trend of PNH RBCs C3d+ during therapy with ECU and RAVU of Florence's court [N = 7]. On the left of the dotted line: the indicators express the last percentage of PNH RBCs C3d+ after at least 5,5 months of ECU; on the right of the dotted line: the indicators express the percentage of PNH RBCs C3d+ after 6±1-12±1-18±1-24±1-36±1 months respectively after the start of RAVU therapy. In this analysis we included the data of 6 patients from Naples (MM V1, NA02 Sa, CA V2, NA03 E, NA01 C, NA04 Si).

Pair Samples Statistics CD59- C3d+ [Florence and Naples]	Mean	N	Std. Deviation	P value
ECU	15,650	13	10,230	0,0600
RAVU (≥36 months)	20,830	13	13,990	

Table 14.

Figures 17-18, Table 13-14. The levels of C3d do not undergo statistically significant changes in the transition from ECU to RAVU in Florence's court and the data do not change if we included patients from Naples.

In summary, this cytofluorimetric study showed that:

1) the binding of C3 fragments to PNH RBCs has been detected both during RAVU therapy, as well as during ECU therapy, whenever C5 is blocked by accumulation of complement cascade factors upstream of C5: thus, the binding of C3 fragments is not exclusive to ECU therapy;

2) the levels of C3 fragments bound to PNH RBCs are substantially unchanged after initiation of RAVU therapy compared to those seen during ECU therapy: thus, the C3 binding does not vary significantly depending on whether the C5 blockade is established by ECU or RAVU.

4.4. Evaluation on efficacy of therapeutic inhibition of the amplification loop on C3 binding

4.4.1. Case report

A 45-year-old patient in April 2011 suffered from an episode of hemolytic anemia associated with modest pancytopenia, reticolocytosis, reduced haptoglobin and elevated lactate dehydrogenase. The patient complained of deep asthenia and occasionally dark urine, difficulty swallowing and cramping pain in the lower limbs. The presentation and symptomatology were strongly suggestive for PNH, and the diagnosis was confirmed by cytofluorimetric analysis that demonstrated the presence of a PNH population: erythrocytes 12%, granulocytes 64%, monocytes 72%. The large clone and the elevation of lactate dehydrogenase levels (more than three times the normal values) associated with whites within the limits of normal and slightly reduced platelets, were characteristic of a predominantly hemolytic form of PNH. Screening for thrombotic risk did not reveal the presence of the most common predisposing genetic factors (Factor V Leiden, factor II and MTHFR polymorphisms, protein S and protein C). For hemolytic forms of PNH, ECU therapy is indicated, which, after vaccination with tetravalent conjugated anti-meningococcal, began in 2001 and continued regularly with the standard dosage of 900 mg ECU every 14+/-2 days. After initiation of ECU therapy, hemolysis indices returned promptly (lactate dehydrogenase generally around 1.3/1.4 the upper limit of normal), asthenia was reduced and other PNH symptoms disappeared, but hemoglobin levels fluctuated between 6.8 g/dL and 9.2 g/dL so the need for transfusion support continued.
The patient received 29 units of concentrated blood cells in 2015, 31 in 2016, 28 in 2017 and 23 in 2018 of which 12 in 24 weeks, so after the start of therapy with ECU, the patient received an average of 2 units of concentrated erythocytes per month. This transfusion requirement was not associated with clear evidence of bone

marrow failure requiring specific treatment since the PNH clone remained extensive, reticulocytosis persisted (between 150,000-200,000 per microL), and neutrophil levels (approx. 1,000-1,200/microL) and platelets (approx. 70,000-90,000/microL) similar to those observed in many PNH patients. However, during treatment with ECU, the presence of a large population of C3-coated PNH erythrocytes was documented in the patient. This patient is clearly part of that 30% of patients in whom ECU therapy, despite the effective control of intravascular hemolysis and related symptoms, remains dependent on transfusions because a de novo extravascular hemolysis is established, witnessed by persistent reticulocytosis and the presence of a discrete population of PNH blood cells covered with fragments of C3.

It is strange that in this patient the globular volume was normal despite the reticulocytosis. Typically, in PNH patients this happens when reticulocytosis is associated with martial deficiency, in this patient the iron balance was, due to transfusions, unbalanced towards accumulation. The Sardinian origins of the patient led to the suspicion that she was a carrier of beta-thalassemic trait. This suspicion could not be confirmed by the standard methods of hemoglobin separation (electophoresis and/or HPLC) since significant amounts of non-autologous blood cells from transfusions were present in the patient's blood. To solve this problem, we resorted to a molecular approach, which was facilitated by the Patient's Sardinian origins. In patients of Sardinian origin, almost all cases of thalassemia are determined by the nonsense mutation on codon 39 CAG>TAG of the Beta-globin gene. For this reason, we subjected the patient's DNA to amplification of exons I and II of beta globin which, then, we analyzed with the Sanger sequencing technique [Figure 19].

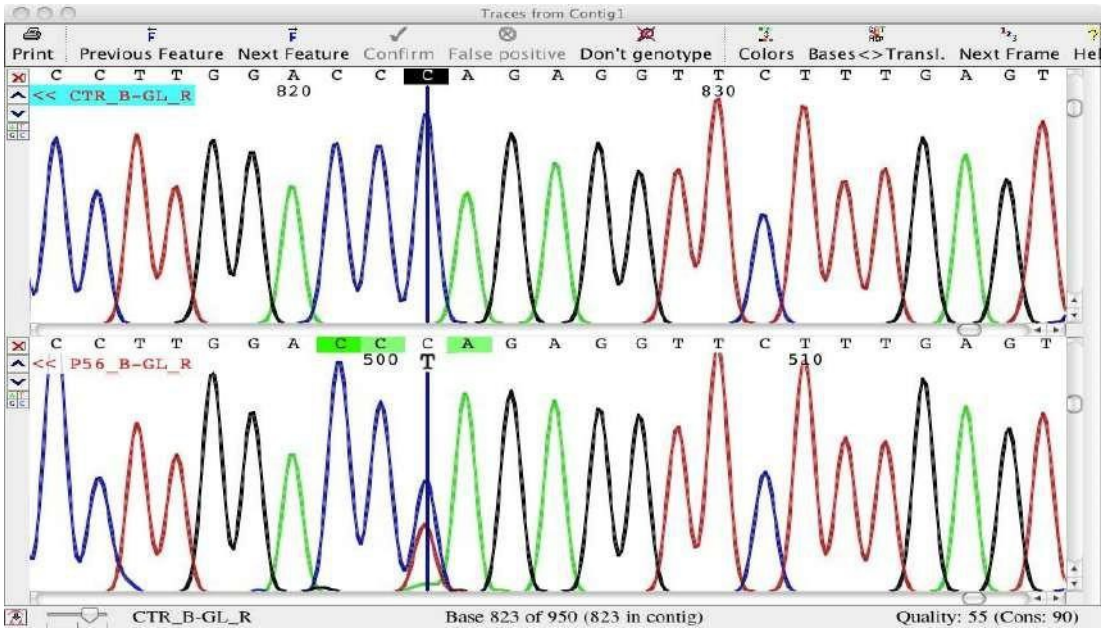


Figure 19. Sanger sequencing of amplified exons I and II of the β-globin gene: electropherogram of codons 36 to 41.

This analysis confirmed the clinical suspicion that the patient was heterozygous for the Beta-zero thalassemic mutation 39. Despite the not very low frequency of thalassemic mutations, only one case of PNH is reported in the literature in a patient carrying a thalassemic trait [Yin XL, 2011].

4.4.2. Clinical trial with Factor D inhibitor

Our center in 2018 was selected for a phase II trial of a factor D inhibitor named ACH-0144471 (danicopan). ACH-0144471, by blocking upstream the activation of C3 via the alternative pathway, could reduce the phenomenon of C3 binding and, consequently, the extravascular hemolysis observed in PNH patients treated with ECU. The trial was reserved for PNH patients requiring transfusion support during ECU therapy and included the addition to ECU therapy of the three-times daily administration of an oral dose of 100 mg danicopan scaled up to 200 mg after 3 months.

The patient described in the previous section met all the inclusion criteria required by the trial. The patient, after signing the consent, extended the prophylaxis of infections by encapsulated bacteria with vaccinations against hemophilus and pneumococcus in addition to those provided for ECU (quadrivalent meningococcal and meningococcal B vaccine) and started a chemoprophylaxis with benzatilpenicillin (1,200,000 IU, im every 4 weeks).

Therapy with the ECU/danicopan combination began in December 2018. The patient's hemoglobin stabilized at around 8 g/dL [Figure 20, a], neutrophils and platelets remained stable and no variations in lactate dehydrogenase levels were observed. The transfusional requirement has almost been eliminated: in the last two years the patient has been transfused only twice receiving two units of concentrated blood cells each time [Figure 20, b], in the first case the transfusion was necessary after an infectious episode (pharmacodynamic breakthrough), while in the second case no potential triggers were identified.

Figure 20. Following the addition of danicopan, there was an increase in hemoglobin levels associated with the abolition of transfusion requirements [a] and a progressive decrease in the percentage of CD59-C3d+ erythrocytes [b].

In this patient we have prospectively studied the binding of C3 to PNH erythrocytes, and we have observed that after the addition of danicopan [Figure 19, b] there is a reduction in C3 binding associated with a parallel reduction in reticulocytosis. These results would confirm the hypothesis that the decrease in the levels of binding of C3 fragments is associated with a proportional attenuation of the phenomenon of extravascular hemolysis with a consequent improvement in the clinical outcome, witnessed by the significant reduction in transfusion needs.

The patient achieved a good clinical response to the combination therapy consisting of ECU and danicopan, evidenced by the fact that the patient achieved the almost complete abolition of the transfusion requirement after the addition of the experimental drug. However, the patient did not achieve the normalization of hemoglobin levels that was observed in almost all other patients enrolled in this study **[**Kulasekararaj AG, 2019].

The patient's response to ECU/danicopan combination therapy, although clinically significant, remains inadequate: in fact, in the classification of the unmet need it passes from "minor response" to "partial response", not reaching the highest levels. The factors that can contribute to an inadequate type of response in this case could be multiple:

1) The patient has a background of bone marrow insufficiency which, although not uncommon in hemolytic PNH patients, could affect the degree of response despite the discrete reticulocytosis.
2) The patient is heterozygous for β-thalassemia. This condition, which generally manifests itself with modest levels of anemia, could make hematopoiesis less efficient in responding to the hemolytic stress present in PNH. There are no specific studies on the interaction between PNH and thalassemia, but it is interesting that recently it has been shown that the glucose-6-phosphate-dehydrogenase deficiency associated with PNH can interfere with the response to ECU through an increase in oxidative stress [Sica M, 2020].

3) The binding of C3 to the patient's PNH erythrocytes has been reduced, but has not disappeared and this is associated with the persistence of reticulocytosis. These data suggest that in this patient danicopan, administered every 8 hours, may not be able to completely block her target. This could result from pharmacodynamic and/or pharmacokinetic problems, but remains to be investigated.

Most likely, the combination of all these mechanisms contributes to a lower level of response to ECU/danicopan combination treatment in this patient than that observed in the other patients enrolled in the study.

4.5. Investigation on the mechanism of PNH hemolytic crisis induced by bacteria

4.5.1. Assessment of bacteria's growth in human sera

We first tested via OD600 the bacterial growth both in the pooled normal human sera (S) and in the pooled heat-inactivated human sera (iNHS) of 20 different subjects and AB0 groups after overnight incubation [Figure 20]. The analysis showed [Figure 21] that bacteria were able to replicate both in the NHS and in the iNHS. The growth analysis resulted increased with statistical significance in the iNHS compared to control (OD600 fold 2 vs. 1; $p < 0,05$ in triplicate experiments). These results suggest that when the activation of complement cascade is blocked such as in the iNHS, bacteria can double-replicate than in the S, in which the complement cascade is able to activate and kill pathogens.

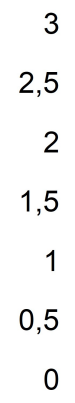


Figure 21. Evidence of bacteria's growth in normal human sera ($p < 0.01$).

4.5.2. Assessment of potential “bystander” lysis of PNH RBCs after co-incubation with bacteria

A significant percentage of PNH patients despite undergoing anti-complement therapy continue to experience hemolytic crises that are defined “breakthrough hemolysis” (BTH). It would be expected that with blockade of complement activation by complement inhibitors, BTH would be abolished, instead they occur whenever amplification of complement activation is induced by conditions of different nature that are associated with a higher density of C3b such as: infections, surgery and/or pregnancy etc. Thus, in this report, we investigated whether PNH red blood cell hemolysis occurs under conditions where CP, LP and/or AP are activated by E. coli in vitro. Our goal was to determine if the mechanism involved is the "bystander" hemolysis due to massive pathogen-driven complement activation. Our hypothesis is that the resulting C3b binds PNH RBCs and results in complement-mediated hemolysis through amplification of the alternative pathway.

4.5.3. PNH RBCs are more susceptible to complement-mediated lysis when co-incubated with bacteria

We assessed the lysis of washed PNH RBCs from 12 PNH patients both in the A, B, 0 type-matched NHS and in the A, B, 0 type-matched iNHS by spectrophotometry after overnight incubation. The lysis of PNH RBCs resulted significantly increased after comparing the lysis rate of a healthy donor RBCs in the same experimental conditions. Moreover, no lysis of PNH RBCs was assessed in iNHS samples. These results demonstrate that PNH RBCs are more susceptible to lysis than healthy donor RBCs when co-incubated with bacteria. Furthermore, the absence of PNH RBCs lysis in iNHS samples demonstrate that lysis is complement-mediated [Figure 22].

Figure 22. PNH RBCs showed more trend to lysis when co-incubated with bacteria [$p < 0.01$). S= normal human sera; iNHS= inactivated human sera. E. coli= Escherichia coli.

4.5.4. PNH RBCs are more susceptible to complement-mediated lysis when co-incubated with bacteria after addition of Mg^{+2}

Since alternative pathway has a magnesium-dependent step, the binding of B to C3b to form the C3 convertase, that is absolutely-dependent for the surface phase and relative-dependent for the fluid phase [James K, 1982], we assessed the PNH RBCs lysis after addition of Mg^{+2} in order to favorite the activation of alternative pathway after co-incubation with E. coli by using both NHS and iNHS in the same experimental conditions. Then, we analyzed the rate of hemolysis by spectrophotometry. The results showed that PNH RBCs lysis is significantly increased when the concentration of Mg^{+2} is optimized confirming the involvement of alternative pathway in PNH RBCs lysis after incubation with E. coli [Figure 23].

Figure 23. The pontential bystander lysis of PNH RBCs lysis is significantly increased with optimized concentration of Mg^{+2}. S= normal human sera; E. coli= Escherichia coli; Mg= magnesium.

4.5.5. MgEGTA-chelated sera showed no effect on potential bystander hemolysis of PNH RBCs when co-incubated with E. coli

Magnesium (Mg) and calcium (Ca)-divalent ions are required for efficient complement activity [Des Prez RM, 1975; Janeway CA Jr, 2001]. The alternative pathway has a Mg-dependent step: the binding of B to C3b to form the C3 convertase. The classical pathway has a Ca-divalent ions-dependent step and a Mg-dependent step: C1q is a calcium-dependent sugar-binding protein, a lectin, belonging to the collectin family of proteins, which contains both collagen-like and lectin domains hence the name collectin; it has six globular heads, linked together by a collagen-like tail, which surround the $(C1r:C1s)_2$ complex; binding of more than one of the C1q heads to a pathogen surface causes a conformational change in the $(C1r:C1s)_2$ complex, which leads to activation of an autocatalytic enzymatic activity in C1r; the active form of C1r then cleaves its associated C1s to generate an active serine protease that starts the classical pathway; moreover, the magnesium-dependent reaction in the classical pathway is represented by the enzymatic action of C1s on C4 and C2.

EGTA is a divalent-ions chelator and it is also able to chelate Ca-divalent ions in human sera necessary for the activation of classical pathway; its salt MgEGTA prevents activation of the classical pathway by chelating Ca-divalent ions and spontaneously activates the alternative pathway [Fine DP, 1977].

In order to investigate whether classical and alternative pathway are involved in bystander hemolysis, we inoculated the JM 109 E. coli strain by standards method into a pooled normal human serum containing PNH RBCs (S + E. coli) and into a MgEGTA-chelated pooled normal human sera [10 mM] (S + E. coli + MgEGTA) and then, we assessed the percentage of bystander hemolysis by spectrophotometry in both the settings [Figure 23]. As shown below, no significant difference was found if we compare the bystander lysis in MgEGTA-chelated pooled human sera (S + E. coli + MgEGTA) with the control (S + E. coli) ($p > 0.01$). These data suggest that the

classical pathway might have no significant effect on the potential bystander hemolysis. Thus, the alternative is suggested to be the only complement pathway involved in bystander hemolysis including the activation by fluid phase and by the direct activation on the surface of RBCs [figure 24].

Figure 24. The potential bystander lysis showed no significant decrease in MgEGTA-chelated pooled human sera compared to control [p> 0.01]. S= normal human sera; E. coli= Escherichia coli; MgEGTA= magnesium dichloride-ethylene glycol tetraacetic acid.

4.5.6. Analysis of correlation between bystander hemolysis and PNH RBCs clone

In order to investigate if there is a potential correlation between the bystander lysis of RBCs in PNH patient, which include both CD59+ and CD59- RBCs populations, and the PNH clone size in RBCs, we evaluated the bystander lysis in RBCs (%) under the above experimental settings by spectrophotometry and the PNH clone size in RBCs (CD59 - %) by flow-cytometry for each PNH patient of our court. The experiment was repeated in triplicate for each patient.

As shown by the linear regression [figure 25], bystander lysis of RBCs and PNH clone size have a statistically significant linear dependence (R^2= 0.7): as the percentage of the PNH clone size in RBCs increases so increases the percentage of bystander lysis of RBCs. Thus, the increase of bystander lysis of RBCs appears directly correlated to the increase of PNH clone size in RBCs.

These data suggest that only PNH RBCs (CD59-) might be destroyed by the bystander lysis, probably due to their loss of complement inhibitors, on the other hand, the normal RBCs (CD59+) might be spared from the bystander lysis due to the presence on their surface of complement inhibitors (CD59 and CD55). Moreover, we are going to confirm these data by flow-cytometry.

Figure 25. The increase of bystander lysis of RBCs is directly correlated to the increase of PNH clone size in RBCs [R^2= 0,7]. RBCs= RBCs; PNH RBC clone= PNH clone size.

5. Conclusions

Our study shows that treatment with RAVU in PNH patients has been as effective as treatment with ECU, confirming the results obtained in large-scale clinical trials. In addition, the study demonstrates the persistence of unmet clinical needs in RAVU therapy as well as during treatment with ECU. Among the etiopathogenetic mechanisms underlying the these unmet clinical needs it is undoubtedly involved the binding of C3 fragments to PNH erythrocytes of which we would like to investigate in vitro the exact dynamics and all the "actors" involved in order to identify new therapeutic targets. In the light of the knowledge acquired to date, it is expected that the clinical needs unmet by C5 inhibitors can be improved by the use of new inhibitors aimed at the different components of the complement cascade, especially of the proximal phase, designed to prevent, possibly in monotherapy and/or in combination with terminal phase inhibitors, the activation of the mechanisms that affect the optimal functioning of the standardized therapies currently available for PNH patients.

Moreover, bacteria (E. coli) can activate the complement cascade in human serum which explicates its bactericidal effect demonstrating by the reduced bacteria growth compared to the increased bacteria growth in heat-inactivated sera, in which the complement cascade can’t be triggered by bacteria, since the absence of heat-labile activation complement products. After bacteria triggered complement activation, PNH RBCs seem to be more susceptible to complement mediated-lysis compared to healthy control. Furthermore, PNH RBCs show an increased complement-mediated lysis triggered by bacteria, especially when alternative pathway is enhanced by the optimization of magnesium ion concentration and after addition of MgEGTA, that blocks the activation of classical pathway, we didn’t observe any significant change in rate of lysis, suggesting that is the alternative pathway which is the main involved in PNH red blood cell lysis after co-incubation with bacteria.

6. Future prospectives

6.1. Outlook on complement system in PNH

The observation of the clinical outcome of PNH patients enrolled in clinical trials with new inhibitors will provide further evidence on the functioning of the CS. Specifically, we will want to compare the effects of C5 inhibition with the inhibition of the proximal complement pathway (C3, Factor D) in vivo and also in vitro.

Furthermore, the reproduction of an in vitro hemolytic crisis model, in our case induced by bacteria, could represent a starting point for better understanding how the amplification of complement activation and its actors interact with PNH RBCs, determining their lysis. More specifically, we would like to know whether the bystander mechanism, which we proved in this study, is mediated by complement factors present in the fluid phase or by the direct activation of the complement on the surface of PNH RBCs.

The combination of the results of the in vitro studies with clinical data will provide the basis for the development of new complement inhibition strategies aimed at improving the clinical management of patients with PNH.

6.2. Outlook on potential clinical implications of iPSCs in PNH

iPSCs are useful in two main applications for the study of PNH:

- modeling of the disease
- generation of HLA-compatible hematopoietic stem cells for transplantation.

6.2.1. Disease's modeling

In 2012, Brodsky's group studied the effects of the lack of the PIGA gene in the process of hematopoiesis and established human induced pluripotent stem cell lines (hiPSCs) that lack PIGA expression and therefore do not yet express GPI. [Brodsky, 2012].
This study demonstrated that hiPSCs lacking PIGA are unable to generate hematopoietic cells and that maturation block occurs concurrently with the generation of CD56-expressing mesodermal progenitors. However, it was still possible to generate negative GPI hematopoietic progenitors in multiple cell lines as a result of the transient expression of the GPI anchor during differentiation and thus, to generate a potential disease model.

6.2.2. Potential source of HSC for autologous transplant

Hematopoietic stem cell transplantation (HSCT) is the only curative therapy for PNH, however, HLA donor mismatches and post-transplant complications limit its use in daily clinical practice. In light of this, patient-induced pluripotent stem cells could represent an interesting source for autologous HSC generation to avoid the negative effects resulting from allogeneic HSCT. In this regard, it should be

emphasized that in PNH mutations involve only the PIGA gene of HSCs and their progeny, therefore other tissues are not affected by this genetic defect and therefore, can be used to produce autologous HSCs to treat patients with PNH without running the risk of complications related to transplantation. The study by Pratumkaew and collaborators [Pratumkaew P, 2021] was the first to derive iPSCs from somatic cells of PNH patients and, more specifically, iPSCs were derived from human dermal fibroblasts, characterized and differentiated into hematopoietic cells. The results of this study represent the promise of a potential source of autologous HSC for patients with PNH. However, we have to remember that in PNH patients is present an autoimmune attack against normal (GPI positive) HSC thus, also with the usage of autologous iPSC derived HSC we will still need a strong immune ablative treatment

6.2.3. Results overview

I have practiced with iPSC related technique by working on an iPSC model used for the study of hematopoiesis maturation and, specifically, to study the role of specific miRNAs.

Hematopoiesis changes over the course of life to meet the needs of maturation and aging. The definitive compartment of hematopoietic stem and progenitor cells (HSPC) is remodeled from gestation to adulthood, a process regulated by the Lin28b / let-7 heterochronic axis which is the most studied regulator of age-specific hematopoiesis [Copley, 2013; Rowe, 2016a, 2016b; Yuan, 2012].

Let-7 comprises a family of microRNAs (miRNAs), i.e. short regulatory RNAs that repress the expression of target messenger RNAs that play important roles in development. In most conditions, the control of their expression occurs both at the transcriptional and at the post-transcriptional level. The miRNA biogenesis pathway involves sequential processing of primary miRNA transcripts (pri-miRNA) by the microprocessor complex (which includes the enzyme RNaseIII Drosha and the double-stranded RNA-binding protein DGCR8) to release 60 -70-nt precursor miRNAs (pre-miRNAs) which are subsequently cleaved by the Dicer complex to produce mature 22 nt miRNAs.

Lin28b is the heterochronic RNA binding protein and is a key regulator of definitive hematopoietic maturation. Lin28 selectively inhibits the maturation of miRNAs of the let-7 family. Furthermore, Lin28b has been shown to regulate globin change, HSC self-renewal, and both myeloid and lymphoid maturation through repression of the let-7 microRNA family and modulation of their downstream targets, as well as through let-7 independent mechanisms [Basak, 2020; Copley, 2013; Rowe, 2016a, 2016b; Yuan, 2012]. Lin28b exerts wide-ranging effects in HSPCs, with its activity sufficient to confer juvenile hematopoiesis in adult cells by inhibiting the stability of let-7 microRNA or by directly regulating translation of specific mRNAs [Basak, 2020; Lee, 2013; Rowe, 2016b]. Lin28b is downregulated during developmental and

maturation progression, releasing let-7 microRNAs to implement adult hematopoiesis [Rowe, 2016b].

In a recent study published by Rowe's group, the let-7 target is implicated in the maturation of HSPC development and it is shown that the Lin28/let7 axis is necessary for effective hematopoietic maturation. The results published in this study have a potential translational impact on the understanding of the mechanisms by which blood disorders are affected by particular ages [Rowe, 2022].

In our study, we used for cloning the lentiviral vector pLKO-Tet-On, which contains all the necessary components for the inducible expression of shRNA in target cells, otherwise in the absence of tetracycline/ doxycycline, shRNA expression is repressed by constitutively-expressed TetR protein. Upon the addition of tetracycline/ doxycycline to the growth media, shRNA expression is triggered resulting in target gene knock-down. pLKO-Tet-On has only 2 cloning sites available: AgeI and EcoRI. We permormed the backbone digestion with those two incompatible enzymes and then, we designed the oligos so that AgeI and EcoRI sites are mutated in the final construct to prevent oligo excision. We cloned our target gene which encodes for let-7g microRNA in the lentiviral vector pLKO-Tet-On and confirm the cloned plasmid by NGS sequencing. Then, in order to confirm the responsiveness of doxycycline-inducible promoter, we perfomed transient transfection in HEK293Ts with lipofectamine and measured the expression of let-7g triggered by doxycycline by RT-qPCR and compared to control without doxycycline [Figures 26; 27, A, B, C].

Expression of Let-7g construct normalized to U6 in HEK293

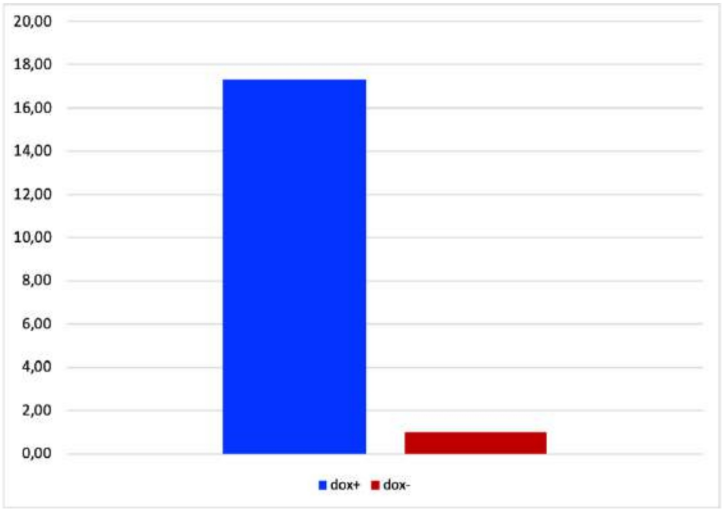
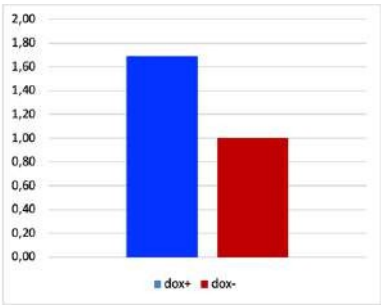


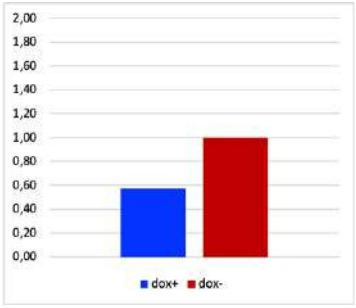
Figure 26.

Let-7g expression in pLKO- Tet-On/Let-7g normalized to U6



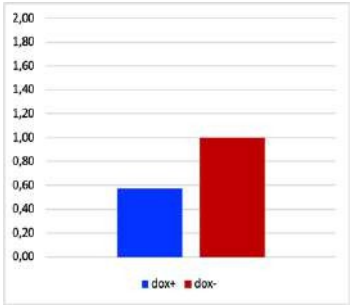
A

Let-7g expression in EBB normalized to U6



B

Let-7g expression in HEK293Ts normalized to U6



C

Figure 27.

Figure 26; 27, A, B, C. Let-7g expression in HEK293Ts compared to controls measured by RT-qPCR.

Next, we transduced the fibroblast-derived iPSCs of a patient with clonal hematological disease by introducing our target gene let-7g and with the empty vector and performed the same procedure for a cell line of fibroblast-derived iPSCs from a healthy subject. Then, we kept the cultures and selected the cell lines of interest using puromycin [Figure 28]. These selected cell lines will allow us to explore the function of the let-7g target gene in the maturation process of hematopoietic cells.

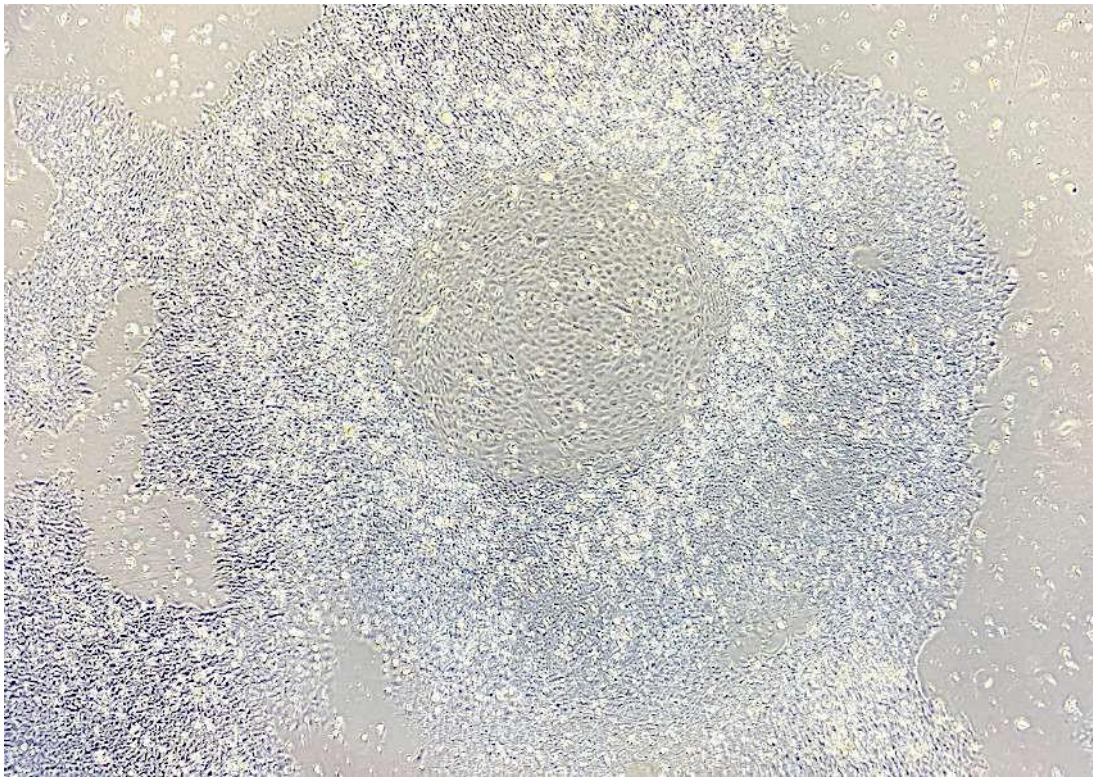


Figure 28. Puromycin-selected iPSCs cell line (C13) after lentiviral transduction with let-7g construct (personal morphology database).

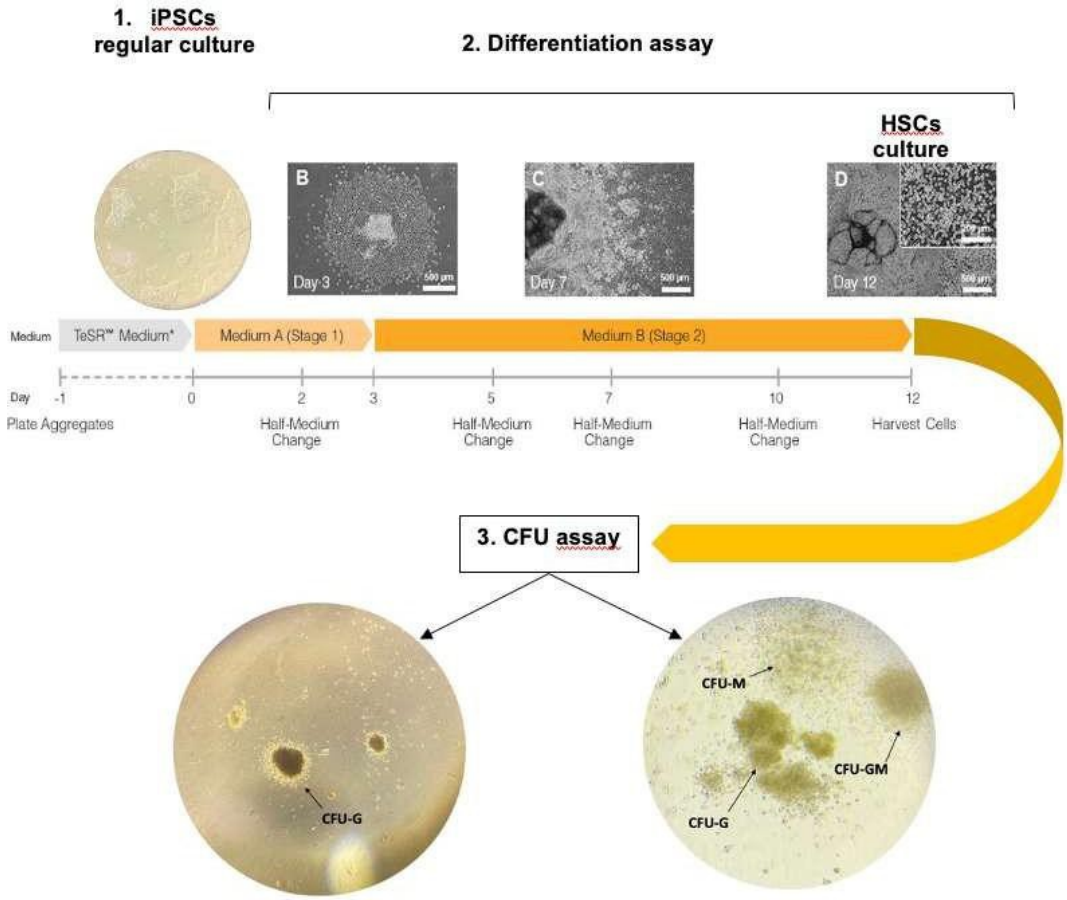


Figure 29. Experimental design of differentiation process from iPSCs to hematopoietic stem progenitor cells. 1. iPSCs colony comprised of highly packed cells and well-defined borders (personal morphology database). B. The differentiation assay results in generation of HSCs from iPSCs. C-D. CFUs culture derived from PSCs induced from fibroblasts of a healthy donor (personal morphology database).

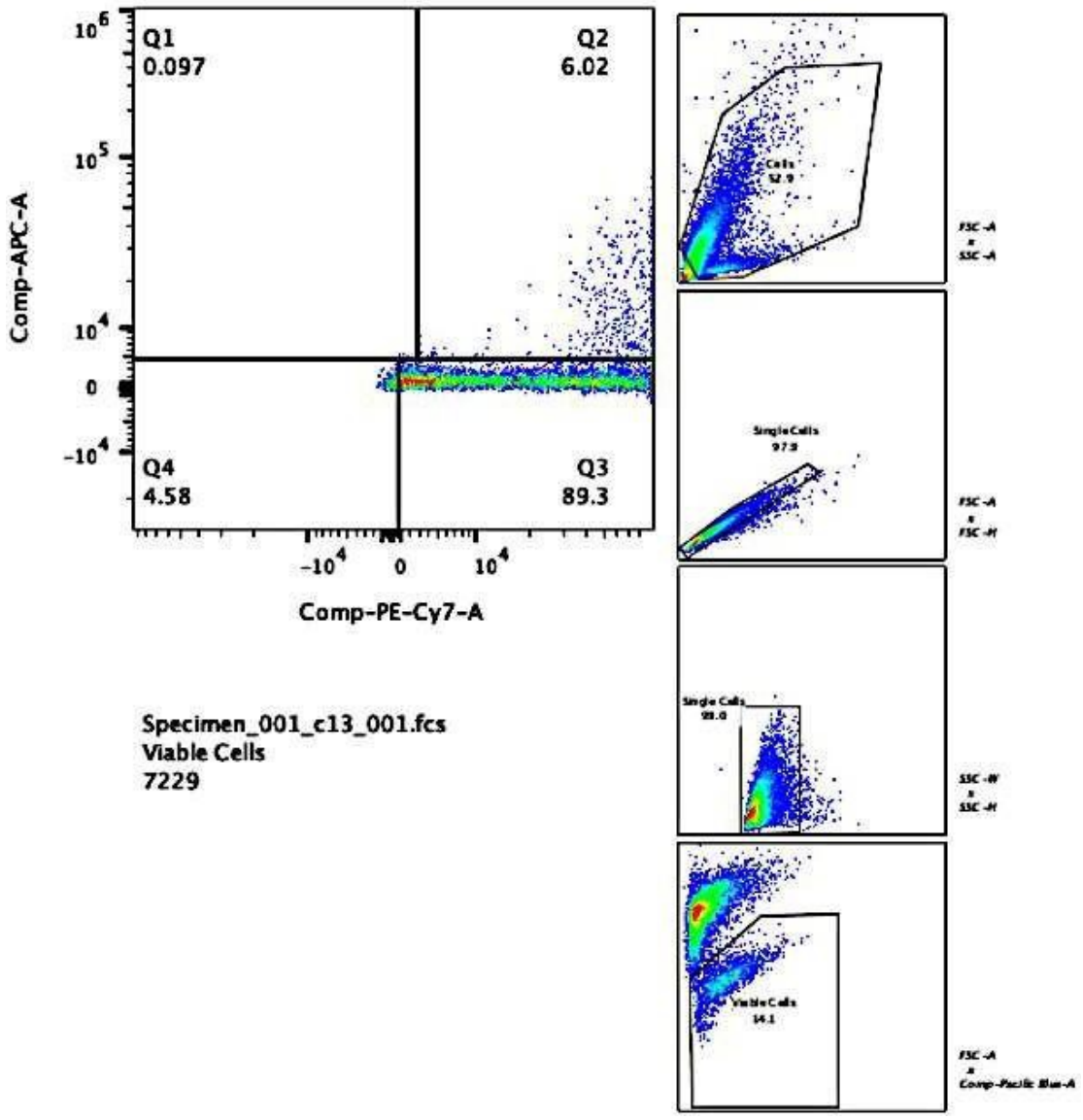


Figure 30. Flow cytometry analysis. After differentiation assay HSCs (C13 cell line) express CD34 (APC antibody) and CD45 (PE-Cy7 antibody) (personal flow-database).

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