

# The importance of flow cytometry in the diagnosis and monitoring of paroxysmal nocturnal hemoglobinuria

## *A importância da citometria de fluxo no diagnóstico e monitoramento da hemoglobinúria paroxística noturna*

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### ABSTRACT

**Introduction:** The paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal disease of the hematopoietic stem cells, and it is clinically characterized by chronic intravascular hemolysis, bone marrow failure and hypercoagulability leading to thrombosis. It is a rare disorder of the hematopoietic stem cells that occurs due to a somatic mutation in the gene phosphatidylinositol glycan class A (PIG-A). **Objective:** Here we reviewed the importance of screening and monitoring of individuals with high risk of developing PNH, since the early diagnosis of the disease is essential for better prognostic and treatment choice for the patient. **Method:** A review was carried out with great focus on the pathophysiology and diagnosis of PNH, mainly with the use of flow cytometry technique to detect the disease. **Results:** This gene codifies an enzyme essential to the formation of glycosylphosphatidylinositol (GPI), which acts as a molecular anchor for many membrane proteins. The alteration of GPI synthesis promotes a partial or complete loss of proteins that needs this molecular anchor to bind to the cell surface. Among these proteins are the CD55 and the CD59, which control the activation of the complement cascade. **Conclusion:** The immunophenotyping exam with flow cytometry is considered the reference test for PNH diagnosis, since the technique is highly sensitive and specific, presenting advantages as the quantitative identification of small populations of cells with PNH phenotype and the capacity to distinguish cells with partial or total deficiency of GPI-anchored proteins.

**Key words:** hemoglobinuria paroxysmal; flow cytometry; immunophenotyping.

### RESUMO

**Introdução:** A hemoglobinúria paroxística noturna (HPN) é uma enfermidade clonal adquirida de células-tronco hematopoiéticas; caracteriza-se clinicamente por hemólise intravascular crônica, falência medular e hipercoagulabilidade, levando a trombozes. É uma rara desordem das células-tronco hematopoiéticas que ocorre devido a uma mutação somática no gene fosfatidilinositol glicano classe A (PIG-A). **Objetivo:** Este trabalho teve como objetivo revisar a importância do rastreamento e monitoramento de indivíduos com alto risco de desenvolvimento da HPN, pois o diagnóstico precoce da doença é essencial para um melhor prognóstico e a escolha do tratamento para o paciente. **Metodologia:** Foi realizada uma revisão com mais ênfase na fisiopatologia e no diagnóstico da HPN. O foco principal da pesquisa foi o uso da técnica da citometria de fluxo para a detecção da doença. **Resultados:** Esse gene codifica uma enzima essencial na formação de glicosilfosfatidil inositol (GPI), a qual atua como molécula âncora de diversas proteínas de membrana nas células hematopoiéticas. A alteração da síntese de GPI gera uma perda parcial ou completa de proteínas que necessitam dessa molécula-âncora para se ligarem à superfície celular. Entre estas proteínas estão o CD55 e o CD59 presente em eritrócitos, que controlam a ativação da cascata do complemento. **Conclusão:** O exame de imunofenotipagem por citometria de fluxo é considerado o teste de referência para diagnóstico de HPN, pois a técnica é altamente sensível e específica, apresentando vantagens como a identificação quantitativa de pequenas populações de células com fenótipo HPN e a capacidade de distinguir células com deficiência parcial ou total de proteínas ancoradas pela GPI.

**Unitermos:** hemoglobinúria paroxística; citometria de fluxo; imunofenotipagem.

## RESUMEN

**Introducción:** La hemoglobinuria paroxística nocturna (HPN) es una enfermedad clonal adquirida de células madre hematopoyéticas; se caracteriza clínicamente por hemólisis intravascular crónica, insuficiencia medular e hipercoagulabilidad, que conduce a trombosis. Es un trastorno raro de las células madre hematopoyéticas que ocurre debido a una mutación somática en el gen fosfatidilinositol-glicano de clase A (PIG-A). **Objetivo:** Este estudio tuvo como objetivo revisar la importancia del cribado y seguimiento de individuos con alto riesgo de desarrollar HPN, pues el diagnóstico precoz de la enfermedad es vital para un mejor pronóstico y la elección del tratamiento del paciente. **Métodos:** Se realizó una revisión con mayor enfoque en la fisiopatología y diagnóstico de la HPN. El foco principal de la investigación fue el uso de la técnica de citometría de flujo para detectar la enfermedad. **Resultados:** Ese gen codifica una enzima esencial en la formación de glicosilfosfatidil inositol (GPI), que actúa como molécula de anclaje para varias proteínas de membrana en las células hematopoyéticas. Cambiar la síntesis de GPI genera una pérdida parcial o total de proteínas que necesitan esta molécula de anclaje para unirse a la superficie celular. Entre esas proteínas se encuentran CD55 y CD59 presentes en los eritrocitos, que controlan la activación de la cascada del complemento. **Conclusión:** La técnica de inmunofenotipificación por citometría de flujo se considera la prueba de referencia para el diagnóstico de HPN, ya que es altamente sensible y específica, presenta ventajas como la identificación cuantitativa de pequeñas poblaciones de células con el fenotipo de HPN y la capacidad de distinguir células con deficiencia parcial o total de proteínas ancladas por GPI.

**Palabras clave:** hemoglobinuria paroxística; citometría de flujo; inmunofenotipificación.

## INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal acquired hematopoietic stem cell disorder, which is clinically characterized by chronic intravascular hemolysis, bone marrow failure and hypercoagulability causing thrombosis – the leading cause of death in the disease, even when treated<sup>(1, 2)</sup>. Fatigue, abdominal pain, chronic kidney disease, dysphagia and erectile dysfunction are symptoms characteristic of the disease and can be attributed to intense intravascular hemolysis and release of free hemoglobin. Hemoglobinuria, despite being present in few patients, can also be considered a characteristic PNH symptom<sup>(3, 4)</sup>. The disease frequently presents with recurrent infections, neutropenia and thrombocytopenia, and can appear in association with other hematological diseases, such as aplastic anemia (AA) and myelodysplastic syndromes (MDS)<sup>(1, 2)</sup>.

PNH is a disease considered rare, with an estimated incidence of 1.3 new cases per one million individuals each year and an average survival of 10 to 15 years after diagnosis. It is also considered underdiagnosed when associated with other entities such as in bone marrow failure<sup>(4)</sup>. It is more common in young adults, but there are reports of cases in both childhood and older age. In Westerners, manifestations of infection and thrombosis are more common, while in Asians, bone marrow failure prevails<sup>(4, 5)</sup>.

The first description of PNH as a clinical syndrome was given in 1882 by Dr. Paul Strübing, who suggested that hemoglobinuria was a result of the abnormal sensitivity of erythrocytes to systemic

acidosis, due to the accumulation of carbon dioxide during sleep. In 1937, Thomas Ham observed that HPN erythrocytes were hemolyzed when incubated with normal acidified serum. This discovery resulted in the first diagnostic test for PNH, known as the Ham test. Then, with the discovery of the alternative complement pathway, the increased sensitivity of the HPN erythrocytes was then attributed to the complement-mediated lysis<sup>(6, 7)</sup>.

Nowadays, flow cytometry (FC) is the most accurate and informative method for the diagnosis and monitoring of PNH, since its high specificity and sensitivity allow the quantitative identification of small populations of HPN cell clones<sup>(4, 8)</sup>. Early diagnosis and continuous monitoring of individuals at high risk for PNH are essential for a better prognosis and treatment choice, and can have a positive impact on long-term patient outcomes<sup>(4, 9)</sup>. Based on the offered context, this work aimed at presenting a review of current concepts in the diagnosis and monitoring of PNH by FC.

## PNH PHYSIOPATHOLOGY

PNH is characterized by non-malignant clonal expansion of one or more hematopoietic stem cells that have acquired a somatic mutation in the phosphatidyl-inositol-glycan class-A (PIG-A) gene, located on the short arm of the X chromosome (Xp22.1) of a pluripotent cell<sup>(10)</sup>. Those mutations cause an early blocking of the synthesis of glycosyl-phosphatidyl inositol (GPI) anchors. GPI is a glycolipid responsible for keeping more than 150

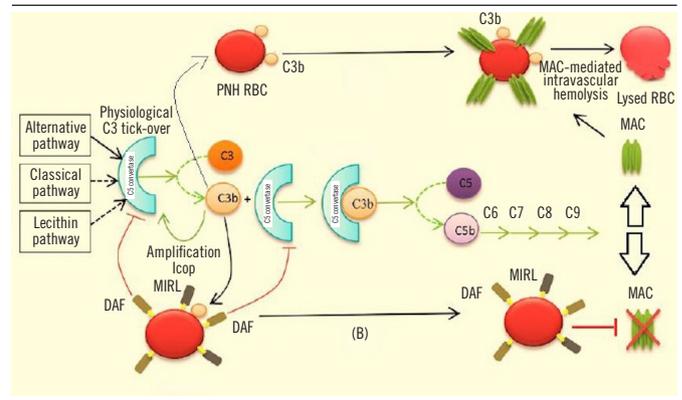
different proteins attached to the plasma membrane of blood cells with specific functions<sup>(1, 2, 11, 12)</sup>.

The reduction in the synthesis of a mature GPI molecule results in the absence of all surface proteins normally anchored by it. Thus, the blood cells from the HPN clone have some degree of deficiency of these proteins, and this degree can be classified as: HPN cells type I (normal), type II (partial GPI deficiency) and type III (total GPI deficiency)<sup>(13)</sup>. The analysis of GPI-anchored proteins on the surface of hematopoietic HPN cells reveals that approximately 40% of sick individuals have a combination of types I, II and III<sup>(14)</sup>.

Among the GPI-anchored proteins are the decay accelerating factor (DAF) and the membrane inhibitor of reactive lysis (MIRL), also known as CD55 and CD59, respectively. Those proteins activate the complement cascade, therefore, PNH hemolysis is a result of the increased susceptibility of these clonal erythrocytes to the complement, by reduction or complete absence of regulatory proteins on the cell surface<sup>(4, 13, 15)</sup>.

The complement system consists of more than 20 serum proteins that interact in a precise manner leading to the generation of products with immunoregulatory, immunoprotective, pro-inflammatory and cytolytic properties. There are three pathways in which the complement is activated: the classic, the lectinin and the alternative pathways. All of them result in the generation of C3-convertase complexes, which mediate the C3 cleavage into C3a and C3b<sup>(13, 16)</sup>.

CD59 interacts directly with the membrane attack complex (MAC) to prevent the formation of lytic pores by blocking C9 aggregation<sup>(17)</sup>, while CD55 accelerates the rate of destruction of C3 convertase<sup>(18)</sup>. In this pathway, CD55 reduces the amount of C3 that is cleaved, and CD59 reduces the number of the MAC that is formed. The alternative way of activating the complement is the center for these mechanisms. In this pathway, the C3 protein spontaneously hydrolyzes and leads to the formation of C3 convertase (a process also known as tick-over). Hemolysis in PNH is chronic due to a continuous state of complement activation by means of tick-over. The mechanism of intravascular hemolysis begins with the increased activity of C3 convertases on the surface of HPN erythrocytes, as a consequence of the lack of CD55. This leads to the activation of C3, C5 and the terminal pathway of the complement, culminating in MAC formation. Under normal conditions, MAC formation is under the regulation of CD59. The absence of CD59 in HPN erythrocytes leads to uncontrolled MAC formation resulting in complement-mediated intravascular hemolysis<sup>(11, 19)</sup>. Of the two proteins, CD59 is the most important in protecting against complement-mediated cell lysis<sup>(20)</sup> (**Figure 1**).



**FIGURE 1** – Complement action in patients with PNH (A) and healthy individuals (B). Due to the presence of MIRL and DAF membrane proteins, normal erythrocytes are protected against activation of the complement system (B). Deficiency of MIRL and DAF makes erythrocytes sensitive to the complement attack, resulting in hemolysis (A)

Adapted from Devalet et al. (2015)<sup>(21)</sup>.

PNH: paroxysmal nocturnal hemoglobinuria; RBC: red blood cells; MAC: membrane attack complex; MIRL: membrane inhibitor of reactive lysis; DAF: decay accelerating factor.

Nitric oxide is the major regulator of vascular physiology, and most of the clinical manifestations of PNH are easily explained by its tissue depletion. Plasma free hemoglobin has great affinity for nitric oxide and removes it from circulation, what can lead to smooth muscle dystonia, platelet activation and aggregation<sup>(3)</sup>.

Tissue depletion of nitric oxide is manifested clinically as asthenia, abdominal pain, esophageal spasm, dysphagia, erectile dysfunction and possibly thrombosis. All of these clinical manifestations are more common in patients with large populations of PNH clones<sup>(2)</sup>. The propensity to thrombotic events can be fatal, since thrombosis occurs predominantly in unusual sites such as supra-hepatic (Budd-Chiari syndrome), portal, splenic and mesenteric veins, or in veins of the central nervous system. The pathophysiology of thrombosis in PNH is not well understood; however, recent studies suggest that there may be a relationship between thrombosis, nitric oxide reduction and hemolysis-induced platelet hyper-reactivity<sup>(22)</sup>.

## PNH CLINICAL CLASSIFICATION

The ability to detect GPI-deficient clones using more sensitive techniques, such as FC, increased the incidence of patients diagnosed with PNH<sup>(4, 23)</sup>. It is observed, however, that the heterogeneity of the disease makes clinical correlation essential, and for a good therapeutic management, both the degree of bone marrow failure and the intensity of hemolysis must be taken into account<sup>(2, 24)</sup>.

In 2005, the International PNH Interest Group (IPIG), based on clinical characteristics, bone marrow characteristics and size of the mutant clone, recognized three subcategories of the disease, emphasizing the presence of hemolysis and/or thrombosis and underlying marrow abnormality (**Table 1**)<sup>(2)</sup>.

TABLE 1 – PNH classification\*

Category	Bone marrow	Intravascular hemolysis	PNH clone
Classical PNH	Hypocellular with areas of erythroid hyperplasia and normal or near-normal morphology	Strong (elevated LDH, often with episodes of macroscopic hemoglobinuria)	Large (50%-100%) GPI-deficient granulocyte population
PNH in the setting of bone marrow disorders	Evidence of a concomitant bone marrow failure syndrome	Mild (often with minimal changes in hemolysis biochemical markers)	Middle-sized (25%-50%) GPI-deficient granulocyte population
Subclinical PNH	Evidence of a concomitant bone marrow failure syndrome	No clinical or biochemical evidence of intravascular hemolysis	Small (< 25%) GPI-deficient granulocyte population

Adapted from Parker (2012)<sup>(15)</sup>.

PNH: paroxysmal nocturnal hemoglobinuria; GPI: glycosylphosphatidylinositol; LDH: lactate dehydrogenase; \*based on the recommendations by the International PNH Interest Group (IPIG)<sup>(2)</sup>.

## PNH DIAGNOSIS

PNH laboratory diagnosis was initially based on tests that showed increased erythrocyte sensitivity to complement-mediated lysis in acid or sucrose-rich medium (Ham and sucrose tests). However, despite the good specificity of these tests, both have low sensitivity and may produce false-negative results in patients with small PNH clones who have recently undergone hemolysis or who have received blood transfusions. Currently, the importance of these tests is just historical: they are no longer recommended in screening for PNH<sup>(2, 4, 25)</sup>.

At present, PNH specific diagnosis and classification are made by detecting GPI-anchored antigens in hematopoietic cells using monoclonal antibodies evaluated by FC in peripheral blood cells. The FC also serves as a tool to measure the PNH clone size. In addition, PNH-specific phenotypes are best established with detailed analyses of the erythrocyte population, complete initial blood count, hemolysis biomarkers [lactate dehydrogenase (LDH), bilirubin, haptoglobin], and iron stores<sup>(26)</sup>.

## Flow cytometry

FC is a method capable of simultaneously measuring multiple parameters of particles or individual cells in suspension, by means of a continuous flow system. The flow cytometer is the necessary equipment for the method, consisting of an optical system formed by a set of lasers that emit light on the analyzed particles. The scattering of light emitted at different angles by these particles can distinguish differences in size and internal complexity, which are captured by forward scatter (FSC) and side scatter (SSC) detectors. Furthermore, the presence of fluorescence detectors in the cytometer allows the use of flow cytometry immunophenotyping, allowing the identification of a variety of cellular antigens, through the emission of light by fluorochromes coupled with specific monoclonal antibodies<sup>(27)</sup>.

The immunophenotyping exam by FC is considered the reference test for the detection of PNH, since the technique is highly sensitive and specific, presenting advantages such as the possibility of analyzing erythrocytes, leukocytes and platelets; the quantitative identification of small populations of cells with PNH phenotype and the ability to distinguish cells with partial or total deficiency of proteins anchored by GPI<sup>(2, 4, 8)</sup>.

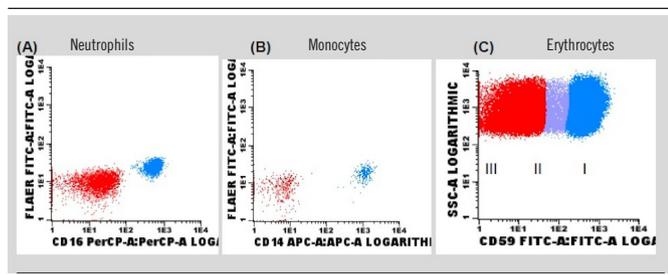
The CD55 and CD59 markers were classically used in erythrocytes to detect PNH clones, because, in addition to being associated with the predominant hemolysis manifestations, they are uniformly distributed in all hematopoietic strains. Monoclonal anti-CD59 and anti-CD55 antibodies specifically bind to GPI-anchored proteins and their absence can be used to detect small populations of PNH cells<sup>(4, 21, 23)</sup>. However, CD55 generally has low intensity of expression in the erythrocyte membrane, not representing a good marker for PNH. Only the analysis of the CD59 antigen on erythrocytes (cells positive for glycophorin A, also known as CD235a) is used to identify the size of the PNH clone in the erythrocytes<sup>(28)</sup>.

Currently, the evaluation of just erythrocytes in routine assays is not considered adequate, as it has little sensitivity due to the short half-life (20-45 days) of circulating HPN erythrocytes, and it is not adequate for detecting small PNH clones (< 1%) in AA and MDS. Also, the hemolytic condition and blood transfusions may underestimate the clone size<sup>(28, 29)</sup>. In contrast, FC analysis of the PNH clone in granulocytes provides a more accurate estimate of the clone size, which is the analysis accepted as the gold standard for PNH diagnosis. Monocyte analysis is generally performed to confirm the results found in granulocytes<sup>(26)</sup>.

The fluorescent labeled aerolysin (FLAER) is a newly developed fluorescent aerolysin reagent, which has been increasingly used in the PNH diagnosis by FC<sup>(21, 30)</sup>. Aerolysin,

which is the main virulence factor produced by the bacterium *Aeromonas hydrophila*, is secreted as proaerolysin and binds selectively and with high affinity to the glycan portion of the GPI anchor<sup>(25, 31)</sup>. One of the advantages of using FLAER is that the assay is not affected by the difference in expression of GPI-anchored proteins in cells that are in earlier maturation stages<sup>(4, 20)</sup>. When FLAER is combined with antibodies against GPI-anchored antigens on granulocytes (CD16/CD24) or monocytes (CD14), it is possible to detect the presence of minimal HPN clones, such as 0.01%. The technological advancement of multiparametric FC also allows the use of immunophenotyping with protein markers not anchored by GPI (for example, CD33 and CD15), to allow the characterization of the cell line when combined with the FLAER reagent<sup>(29, 32)</sup>.

The criterion established for diagnosing and monitoring PNH is evidence of a clonal population of GPI-deficient cells (HPN clone) in at least two different markers (two GPI-linked proteins or one GPI- and FLAER-linked protein) in at least two distinct hematopoietic strains, since there are rare congenital deficiencies of CD55 or CD59 that could be responsible for false-positive results<sup>(4, 8, 29)</sup> (**Figure 2**).



**FIGURE 2** – Immunophenotypic analysis of PNH clones in peripheral blood  
 A) neutrophils were selected for CD45, CD10 and CD64 positivity, and clones were identified for FLAER and CD16 deficiency. In the first graph, we have a 56% PNH clone in neutrophils in red; B) monocytes were selected for CD45 and CD64 positivity. Clones were identified by the absence of FLAER and CD14. In the second graph, we have a 58% PNH clone in monocytes in red; C) erythrocytes were selected for CD235a positivity (glycophorin A) and absence of CD61, CD45 and CD64. PNH clones were identified for CD59 deficiency. The last graph presents three types of cells that we can find in PNH positive samples: cells with complete absence of CD59 expression (type III) in red, cells with weak expression of CD59 (type II) in lilac, and healthy cells with strong expression of CD59 (type I) in blue

Authors' own image.

PNH: paroxysmal nocturnal hemoglobinuria; FLAER: fluorescent labeled aerolysin; FITC: fluorescein isothiocyanate; PerCP: peridinin chlorophyll protein complex; APC: allophycocyanin.

In a study conducted by Brodsky *et al.* (2000)<sup>(33)</sup>, more accuracy of FLAER was observed in the detection of small PNH clones, in comparison with FC assays that use just monoclonal antibodies. Some studies on the granulocyte population observed that the use of FLAER/CD24 combinations provide a more accurate estimate of the HPN clone size<sup>(2, 4, 25, 32)</sup>. In 2014, Sutherland *et al.*<sup>(34)</sup> evaluated the efficiency of a five-color assay in a

single tube using the combination of FLAER, CD157, CD64, CD15 and CD45 to simultaneously detect HPN clones in granulocytes and monocytes. In the results obtained in the study, CD157 showed high levels of sensitivity, demonstrating to have an excellent cost-benefit ratio when compared to the tests that carry out the analysis of granulocytes and monocytes in separate tubes<sup>(34)</sup>.

In 2003, Yang *et al.*<sup>(35)</sup> investigated the performance of several immunophenotypic markers, including the monoclonal antibodies CD235a, CD33, CD15 combined with CD59, CD16, CD24, CD14 and FLAER in a PNH panel using a six-color FC. The obtained results supported the premise that the six-color FC with a PNH panel using the combination of CD59, CD235a, CD33, CD15, FLAER, CD16, CD24 and CD14 can increase and improve the sensitivity of the current methods used in the diagnosis and treatment of PNH, specifically identifying PNH clones in the erythrocyte, granulocyte and monocyte population<sup>(35)</sup>.

## CLINICAL INDICATIONS FOR PNH SCREENING

Early diagnosis is known to be essential for a better prognosis and treatment choice of patients with PNH<sup>(4, 9)</sup>. Therefore, the International Clinical Cytometry Society (ICCS) and the International PNH Interest Group (IPIG) recommend the evaluation and continuous monitoring of populations of patients at high risk for PNH (**Table 2**)<sup>(2, 4)</sup>.

Movalia *et al.*, in 2011, carried out an analysis of the incidence of PNH clones in 6,897 patients with recommendations for tests according to the ICCS and IPIG guidelines<sup>(36)</sup>. FC detected the presence of PNH clones in one out of 16 patients (421/6,897), with the proportion of PNH clones exceeding 1% being one out of 27 patients (255/6,897).

Patients with negative Coombs hemolytic anemia or with concomitant iron deficiency are more likely to have PNH clones<sup>(4)</sup>. Approximately one out of four patients with hemolytic

**TABLE 2** – Clinical indications for PNH tests

Populations at high risk for PNH <sup>*</sup>	Incidence of PNH clone <sup>+</sup>
Negative Coombs hemolytic anemia	22.7%
Hemoglobinuria	18.9%
Aplastic anemia	26.3%
Myelodysplastic syndrome of the refractory anemia type	5.5%
Unexplained cytopenias	5.7%
Unexplained thrombosis (venous or arterial)	1.4%

PNH: paroxysmal nocturnal hemoglobinuria; <sup>\*</sup>based on the guidance of the International Clinical Cytometry Society (ICCS) and the International PNH Interest Group (IPIG)<sup>(2, 4)</sup>; <sup>+</sup>data obtained from the study conducted by Movalia *et al.* (2011)<sup>(36)</sup>.

anemia has these clones<sup>(36)</sup>. Although hemoglobinuria is not present in all patients with PNH, it is a sign of intravascular hemolysis<sup>(2)</sup>, and screening for PNH should be done in any patient with hemoglobinuria<sup>(4)</sup>. Approximately one in five patients with hemoglobinuria has PNH clones<sup>(36)</sup>. At the same time, according to Parker *et al.* (2005)<sup>(2)</sup>, only 26% of PNH patients report hemoglobinuria at presentation.

The relationship between PNH and AA was initially exposed by Dacie (1961)<sup>(37)</sup> and Lewis (1967)<sup>(38)</sup>, and today most individuals with PNH are known to have some evidence of bone marrow failure. Natural history studies have confirmed this association, showing that the prevalence of PNH clone detection in patients with a history of AA can vary between 23%<sup>(24)</sup> and 38%<sup>(39)</sup>. Most patients with this association express only a small PNH clone (< 10%)<sup>(24)</sup>, however, the bone marrow environment can promote the expansion of this clone<sup>(40)</sup>. In a study with 27 AA patients, 48% showed an increase in the PNH clone size. However, the clinical significance of a small HPN cell clone in AA patients remains uncertain. These clones can remain stable or increase and even decrease in size, or may even disappear<sup>(41)</sup>.

MDS are also classic complications of PNH. Currently, with the use of cytometers able to detect GPI-deficient clones of up to 0.003%, the presence of small PNH clones has been observed in up to 20% of patients with low-risk MDS<sup>(13, 42)</sup>. In the analysis by Movalia *et al.* (2011)<sup>(36)</sup>, more than one out of 18 patients with MDS had PNH clones.

Patients with unexplained cytopenias and thromboses are also considered to be at high risk for PNH. Individuals with PNH

are more likely to have a thrombotic complication in unusual locations, including presentations such as Budd-Chiari syndrome or cerebral thrombosis. Thus, unusual presentations of thrombosis should justify screening for PNH<sup>(4)</sup>.

## CONCLUSION

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Although PNH is a rare hematological disorder, it is progressive and potentially fatal, and it is essential to track and monitor individuals at high risk of developing this disease. Accurate diagnosis of PNH is imperative and has significant clinical implications for the treatment and prevention of adverse events. FC immunophenotyping is the most sensitive and informative test available for the diagnosis of PNH, being suitable for the investigation of small subclinical clones that are often present in patients with AA and MDS. The presence of these subclinical clones in patients with AA and MDS may show prognostic and therapeutic implications.

However, there are a number of important considerations in terms of antibody analysis and selection strategies that must be observed. Equally important, result interpretation requires detailed knowledge of the cellular distribution of GPI-linked antigens and their expression in the different stages of differentiation of hematopoietic cells. Clear reports are essential for taking appropriate clinical decisions, and positive laboratory findings must be seen in the context of the disease clinical presentation. The physician's role is to classify and define the most appropriate treatment for each patient.

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