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Laboratory studies for paroxysmal nocturnal hemoglobinuria, with emphasis on flow cytometry

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ABSTRACT

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare acquired clonal hematopoietic stem cell disorder caused by somatic mutations in the *PIG-A* gene, leading to the production of blood cells with absent or decreased expression of glycosylphosphatidylinositol-anchored proteins, including CD55 and CD59. Clinically, PNH is classified into three variants: classic (hemolytic), in the setting of another specified bone marrow disorder (such as aplastic anemia or myelodysplastic syndrome) and subclinical (asymptomatic). PNH testing is recommended for patients with intravascular hemolysis, acquired bone marrow failure syndromes and thrombosis with unusual features. Despite the availability of consensus guidelines for PNH diagnosis and monitoring, there are still discrepancies on how PNH tests are carried out, and these technical variations may lead to an incorrect diagnosis. Herein, we provide a brief historical overview of PNH, focusing on the laboratory tests available and on the current recommendations for PNH diagnosis and monitoring based in flow cytometry.

1. Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare acquired clonal hematological disorder caused by somatic mutations in the phosphatidylinositol glycan, class A (*PIG-A*) gene in the hematopoietic stem cells [1,2]. Previous studies have identified the concurrence of *PIG-A* mutations with other genetic defects, suggesting a stepwise evolution like that observed in other hematological disorders, although this hypothesis still needs to be proved [3].

PIG-A mutations lead to the production of blood cells with absent or decreased expression glycosylphosphatidylinositol (GPI) -anchored proteins (GPI-AP), from which the first to be described and the best characterized are the complement-regulatory proteins decay accelerating factor (DAF, CD55) and membrane inhibitor of reactive lysis (MIRL, CD59).

CD55 (DAF) is widely distributed in blood, endothelial and epithelial cells and it inhibits the complement cascade at the level of the C3 convertase [4,5]. CD59 (MIRL) is expressed in all blood cells, endothelial cells, and cells of the nervous system, and prevents C9 from polymerizing, thereby restricting the formation of the membrane attack complex (MAC) [4,5]. Both proteins play an important physiological role in protecting the cells from complement-mediated damage, and they have been implicated in various pathological conditions. Besides CD55 and CD59, many other GPI-AP are expressed in blood cells, where they function as enzymes, receptors and

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adhesion molecules, being also involved in signal transduction (Table 1). For detailed information about these GPI-AP, including the correspondent receptors or ligands, protein and gene names, function and expression in normal and PNH cells, please see Table 2.

Deficiency of GPI-AP may explain the increased sensitivity of the red blood cells (RBC) to complement-mediated lysis, as well as the main clinical findings of PNH, i.e., hemolysis and thrombosis. Various degrees of bone marrow (BM) failure and dominance of the PNH population may also be linked to the biology and function of GPI-AP, even though the physiopathology is not completely understood [6–8]. A close association between PNH and the BM failure syndromes, such as aplastic anemia (AA), and the fact that auto-reactive GPI-specific T cells have been identified in PNH and in AA, would suggest an immune-mediated mechanism by which GPI-AP deficient cells are resistant to the attack of cytotoxic T lymphocytes, enabling them to emerge [9–11].

Clinically, PNH is being classified, by suggestion of the *International PNH Interest Group* (IPIG), into classic PNH, PNH in the setting of another specified BM disorder, and subclinical (asymptomatic) PNH [12]; the clinical manifestations and the size of the PNH population varies in these PNH subtypes [12]. An international, observational and prospective registry study on PNH (International PNH Registry, ClinicalTrials.gov Identifier: NCT01374360), started by January 2007. This study, which as of July 2017 had enrolled 4948 patients, is the largest repository of PNH patient data and provides valuable information on the clinical characteristics, disease burden, and treatment modalities [13–18]. Other observational, retrospective or prospective, single-center or multicenter studies have been conducted over the world, all contributing to our knowledge of the diagnosis, clinical manifestations, treatment and prognosis of PNH [19–22].

Before the 90s, diagnosis of PNH was made using complement-based hemolysis assays (Ham's test and Sucrose hemolysis test). Nowadays, flow cytometry (FCM) has become the gold standard assay, as it allows to evaluate different populations of blood cells, lets for a precise quantification of the size of the PNH clones and for their characterization, and has a higher sensitivity to detect small PNH populations [23,24].

In 2010, Borowitz et al., on behalf of the *International Clinical Cytometry Society (ICCS)*, proposed the guidelines for the diagnosis and monitoring of PNH by FCM [25]. In the same year, the *Spanish group for PNH study by flow cytometry* gave general recommendations for PNH diagnosis [26]. Two years later, Sutherland et al. suggested specific guidelines for high-sensitivity PNH assays [27]. More recently, the *PNH working group of the Spanish Society for Haematology and Hemotherapy* published a consensus statement for the diagnosis and treatment of PNH [28]. Despite these guidelines, evidence from the *UK National External Quality Assessment Service for Leukocyte Immunophenotyping* (UK NEQAS LI) program revealed major discrepancies on how PNH testing is performed, as well as inappropriate laboratory practices [29]. These technical variations could lead to incorrect diagnoses, highlighting the need for a better understanding, as well as a proper implementation of consensus recommendations [29]. Recently (2018), the ICCS and the *European Society for Clinical Cell Analysis* (ESCCA) established new guidelines for high-sensitivity FCM detection of PNH and published four related manuscripts addressing different issues: evaluation of a patient with suspected PNH or other BM failure disorder, with emphasis on the contribution of FCM for PNH diagnosis, classification and monitoring [30]; assay optimization and reagent selection [31]; data analysis and report [32]; and test validation and quality assurance [33].

This review provides a historical perspective and presents an overview of PNH, focusing on the laboratory tests available, and on the current recommendations for PNH diagnosis and monitoring based in FCM.

Table 1
GPI-anchored proteins expressed on blood cells already in use or potentially useful for the diagnosis of PNH by flow cytometry.

Cluster of differentiation	Blood cells	Tested for PNH diagnosis
CD14	Monocytes	+++
CD16[a]	Neutrophils	+++
CD24	Neutrophils	+++
CD48	Lymphocytes	++
CD52	WBC	+
CD55	WBC, RBC, Platelets	+++
CD58[b]	WBC, RBC	+
CD59	WBC, RBC, Platelets	+++
CD66b	Neutrophils	++
CD66c	Neutrophils, Eosinophils	+
CD73	Lymphocytes	+
CD87	Neutrophils, Monocytes	++
CD108[c]	Monocytes, Lymphocytes	Not tested
CD109[c]	Monocytes	Not tested
CD157	Neutrophils, Monocytes	++
CD177	Neutrophils, Monocytes (?)	+

Abbreviations: GPI, glycosylphosphatidylinositol; WBC, white blood cells; PNH, Paroxysmal Nocturnal Hemoglobinuria; RBC, red blood cells.

[a] Two isoforms: non-GPI linked integral membrane protein (CD16a, expressed mainly in NK cells) and GPI-linked (CD16b, expressed mainly in neutrophils).

[b] Two isoforms: non-GPI linked integral membrane protein (expressed in WBC) and GPI-linked protein (expressed in WBC and RBC).

[c] No published studies were found that tested these antibodies for the diagnosis of PNH.

Table 2
Detailed information about the GPI-anchored proteins expressed on hematopoietic cells.

CD	Normal blood cells								Specification	Protein [Gene] (Chromosome)	Family	Ligand, receptor, enzymatic activity	Main function	References[a]
	NEUT	EOSI	MONO	LYMP	DEND	RBC	RETI	PLT						
CD14	–	–	+	–	–	–	–	–	Monocytes.	CD14 MCSRP *[CD14]* (5q31)	Leucine-rich proteins.	Co-receptor for LPS, along with TLR4 and MD-2.	Monocyte activation and adhesion.	A[72,134] B[77,86, 101,113]
CD16b	+	–	–/+	–/+	–/+	–	–	–	CD16a: CD56 + lo NK cells; T cell subsets; monocyte subsets; DC subsets CD16b: Neutrophils.	CD16 FcγRIIIA FcγRIIIB *[FCGR3A]* *[FCGR3B]* (1q23)	Class III Fc gamma receptors (CD16a and CD16b). CD16a: Integral membrane protein. CD16b: GPI-AP.	Low-affinity Fc receptor for IgG.	CD16a: Responsible for ADCC and involved in phagocytosis. CD16b: Trapping of IgG immunocomplexes.	A[72,97, 135,136] B[77,81, 101]
CD24	+	–	–	–/+		–	–	–	Neutrophils; B cells. Neurons; myocytes.	CD24 HSA *[CD24]* (6q21)	CD24 family. Syaloglycoprotein, Mucin-like molecule.	Binds to P-Selectin, CD171, and other ligands depending on the cellular context	Cell adhesion. Signal transduction. Presynaptic maturation and function. Inhibition of neural growth.	A[72,137] B[77,86, 101,113]
CD48	–	–	+	+	–/+	–	–	–	Lymphocytes, monocytes; DC subsets (myeloid). Endothelial cells.	CD48 BLAST-1 SLAMF2 *[CD48]* (1q23)	SLAM family. CD2 subfamily of the Ig superfamily.	Binds primarily to CD244 (2B4). Low affinity ligand for CD2.	Involved in cell activation.	A[72,138] B[77,139]
CD52	–/+	–/+	+	+	–/+	–	–	–	Lymphocytes, except CD56 + hi NK cells; monocytes; DC subsets. Neutrophils are CD52-/+lo. Male genital tract.	CD52 He5 CAMPATH1H *[CD52]* (1p36)		Binds SIGLEC10, an ITIM –bearing sialic acid-binding lectin.	Involved in complement-mediated cell lysis and ADCC. Involved in sperm maturation.	A[72,140, 141] B[66]
CD55	+	+	+	+	+	+	+	+	RBC (lower intensity than CD59), WBC (higher intensity than CD59). Epithelial cells; endothelial cells.	CD55 DAF CROM Crommer blood group. *[CD55]* (1q32)	RCA family.	Binds to CD97 is a seven-span transmembrane (7-TM) protein that is expressed by leukocytes early after activation.	Accelerates the decay of C3 convertases, C4aC2a and C3bBb, of complement pathway. Protect cells from complement mediated cytolysis.	A[4,5,72] B[81]
CD58	+	+	+	–/+	–/+	+	+	–	Neutrophils; monocytes; lymphocytes (T cells); DC subsets (myeloid); RBC. B cells and plasmacytoid DC do not express CD58.	CD58 LFA-3 *[CD58]* (1p13)	CD2 subfamily of the Ig superfamily.	Ligand for CD2, expressed on T cells and NK cells.	Involved in cell adhesion and CTL-target cell conjugate formation.	A[72,142, 143] B[63,70, 77]
CD59	+	+	+	+	–/+	+	+	+	RBC (higher intensity than CD55). WBC (lower intensity than CD55). Endothelial cells; peripheral and central nervous system.	CD59 MIRL MAC-I Protectin. *[CD59]* (11p13)	RCA family.	Binds to C8 and C9 complement factors of the MAC. Second ligand for the CD2 molecule, expressed on T cells and NK cells.	Prevents the terminal polymerization of the MAC. Protect cells from complement mediated cytolysis.	A[4,5,72] B[76,77]

(*continued on next page*)

Table 2 (*continued*)

CD	Normal blood cells								Specification	Protein [Gene] (Chromosome)	Family	Ligand, receptor, enzymatic activity	Main function	References[a]
	NEUT	EOSI	MONO	LYMP	DEND	RBC	RETI	PLT						
CD66b	+	+	–	–	–	–	–	–	Neutrophils, eosinophils.	CD66b CEACAM8 *[CEACAM8]* (19q13)	CEACAM family (CD66a-d molecules).	The ligands of CD66b are CD66c, CD66e, and Galectins (Galectin-3).	Activation related molecule (increases following stimulation). Mediates interactions between neutrophils and endothelial cells. Involved in neutrophil and eosinophil activation, cell adhesion and migration.	A[72,144,145] B[77,81]
CD66c	+	–	+	–	–	–	–	–	Neutrophils; monocytes. Endothelial cells. Epithelial cells.	CD66c CEACAM6 *[CEACAM6]* (19q13)	CEACAM family (CD66a-d molecules).	The ligands of CD66c are CD66a-e, CD62E (E-Selectin) and Galectins.	Activation related molecule (increases following stimulation). Mediates interactions between neutrophils and endothelial cells. Involved in neutrophil activation, cell adhesion and migration.	A[144,146] B[67]
CD73	–		+	–/+		–		–	Lymphocyte (B and T) subpopulations; Endothelial cells. Cell from various tissues. Overexpressed in many types of cancer.	CD73 NT5E Ecto-5'-nucleotidase *[NT5E]* (6q14)	5'-nucleotidase family.	Ectonucleotidase	Catalyzes the conversion of extracellular to membrane-permeable nucleosides (AMP breakdown to adenosine). Anti-inflammatory and immunosuppressive effects.	A [147–149] B[65]
CD87	+		+	–	–/+	–	–	–	Neutrophils; monocytes.	CD87 uPAR UPAR *[PLAUR]* (19q13)	Ly6/neurotoxin receptor family.	Binds primarily to urokinase.	Converts plasminogen to plasmin. Involved in fibrinolysis, cell adhesion and migration.	A[72, 150–154] B[153,154]
CD108			+		–/+	+			Monocytes; lymphocytes; RBC. Neural cells.	CD108 Sema7A SEMAL JMH blood group antigen (RBC). *[*SEMA7A*]* (15q24)	SEMA family.	Erythrocyte receptor for the *Plasmodium falciparum* MTRAP.	Promotes axon outgrowth. Induces monocyte activation. Influences T cell responses (e.g. proliferation and cytotoxic differentiation). Reduces the production of megakaryocytes and platelets. Interacts with beta1-integrins and plexins. Activates the MAPK pathway.	A[155,156] B No references
CD109	–/+	+	+	–/+	+	–	–	+	Monocytes; DC subsets (myeloid); eosinophils; platelets. HSC subsets. Endothelial cells.	CD109 HPA-15/Gov antigen (platelets) 150 KDa TGF-Beta-1-Binding Protein *[CD109]* (6q13)	Alpha2-macroglobulin/ complement (C3, C4, C5) family.	Binds to TGF-β.	Negatively regulates signaling by TGF-β. Serine-type endopeptidase inhibitor activity.	A[72,157] B No references

(*continued on next page*)

Table 2 (*continued*)

CD	Normal blood cells								Specification	Protein [Gene] (Chromosome)	Family	Ligand, receptor, enzymatic activity	Main function	References[a]
	NEUT	EOSI	MONO	LYMP	DEND	RBC	RETI	PLT						
CD157	+	–	+	–	–	–	–	–	Neutrophils; monocytes.	CD157 BST-1 *[BST1]* (4p15)	CD38 NADase/ADP-ribosyl cyclase gene family .	Binds to extracellular matrix proteins such as fibronectin, fibrinogen, laminin and collagen type I. (Ectoenzyme: ADP-ribosyl cyclase 2).	Adhesion/signaling molecule with enzymatic activity. Involved in cell adhesion and migration (neutrophils and other cells).	A[72,158, 159] B[74,105, 112]
CD177	−/+	–	−/+	–	–	–	–	–	Neutrophil subpopulation, (bimodal distribution); monocytes (to be confirmed).	CD177 PRV-1 NB1 HNA-2a (neutrophils) *[CD109]* (19q13)	Ly-6 superfamily	Binds to PECAM-1/CD31, expressed on platelets and endothelial cells.	Mediates interactions between neutrophils and endothelial cells and is involved in neutrophil activation and transmigration. Co-localizes with proteinase-3, which cleaves the thrombin receptor, decreasing thrombin-mediated platelet activation. Mutations resulting in CD177 overexpression are associated with MPN (PV and ET). Autoantibodies against CD177 may be involved in pulmonary transfusion reactions, and in Wegener's granulomatosis.	A [160–162] B[48]

Abbreviations: ADCC, Antibody-dependent cellular cytotoxicity; ADP, Adenosine diphosphate; AMP, Adenosine monophosphate; BLAST-1, B-lymphocyte activation marker; BST-1, Bone marrow stromal cell antigen-1; CD, Cluster of differentiation; CEACAM, Carcinoembryonic antigen-related cell adhesion molecule; CTL, Cytotoxic T lymphocytes; DAF, Decay Accelerating Factor; DEND, Dendritic cells; EOSI, Eosinophuls; ET, Essential Thrombocythemia; GPI, glycosylphosphatidylinositol; He5, Human Epididymis-Specific Protein 5; HNA, Human Neutrophil Antigen; HPA, Human Platelet Antigen; HSA, Heat stable antigen; HSC, Hematopoietic stem cells; ITIM, Immunoreceptor tyrosine-based inhibitory motif; JMH, John-Milton-Hagen blood group antigen; Ly, Lymphocytes; Ly-6, Leukocyte Antigen 6; LYMPH, Lymphocytes; LFA-3, Lymphocyte function-associated antigen 3; LPS, bacterial lipopolysaccharide; MAC, Membrane attack complexes; MAC-I, MAC inhibitor; MCSLRP, Myeloid Cell-Specific Leucine-Rich Glycoprotein; MIRL, Membrane inhibitor of reactive lysis; MONO, Monocytes; MPN, Myeloproliferative neoplasms; MTRAP, merozoite thrombospondin-related anonymous protein; NAD, Nicotinamide adenine dinucleotide; NEUT, neutrophils; NT5E, Ecto-5'-nucleotidase; PECAM-1, Platelet endothelial cell adhesion molecule-1; PIG-A, Phosphatidylinositol N-acetylglucosaminyltransferase subunit A; PLT, Platelets; PNH, Paroxysmal Nocturnal Hemoglobinuria; PRV-1, Polycythemia Rubra Related protein type 1; PV, Polycythemia Vera; Rt, Reticulocytes; RBC, Red blood cells; RCA, Regulators of complement activation; RETI, Reticulocytes; SEMA, Semaphorin; SLAM, Signaling lymphocytic activation molecule; SIGLEC, sialic acid-binding lectin; Sema7A, Semaphorin 7A; SLAMF2, Signaling lymphocytic activation molecule 2; TGF, Transforming Growth Factor; TLR, Toll-like receptor; uPAR, Urokinase-type plasminogen activator receptor.

CD58 (LFA-3): This molecule is expressed on the cell surface in both a transmembrane and a GPI-anchored form; RBC only express the GPI-anchored form. Some studies have shown that anti-CD58 normally bound to CD55/CD59 deficient WBC and platelets, suggesting that CD58 is expressed as a transmembrane protein in these cells [70].

CD66b: Previously designated CD67.

Other GPI-AP: Enzymes (red cell acetylcholinesterase; neutrophil alkaline phosphatase); Red cell antigens (Holley Gregory, Dombrock, YT); Platelet antigens (GP500 and GP175); Neutrophil antigens (NB1/NB2); Anticoagulant substances: heparan sulfate; TFPI (Tissue factor pathway inhibitor); Other molecules (ULBP/UL-16 binding proteins; PRV-1/Polycythemia rubra vera 1; TRAIL R-3/Trail receptor III).

[a] A) Molecule function and expression in normal cells. B) Expression in PNH cells.

2. Clinical manifestations and disease outcome

Patients with PNH may present with a variety of signs and symptoms, including intravascular hemolysis, hemolytic anemia, neutropenia, thrombocytopenia, renal failure, arterial and pulmonary hypertension, smooth muscle dystonia, infections and thrombosis, among others. Classic PNH typically manifests as intravascular hemolysis with or without hemolytic anemia. Bone marrow failure syndromes that may coexist with PNH include aplastic anemia (AA), AA-PNH, and myelodysplastic syndromes (MDS), MDS-PNH [34–40].

PNH patients always have some degree of intravascular hemolysis, as revealed by biochemical markers – serum lactate dehydrogenase (LDH), unconjugated bilirubin, and haptoglobin –, and variable degrees of hemoglobinuria and hemosiderinuria, although not all patients have anemia and episodes of dark urine [12]. Hemolysis in PNH is chronic because of a continuous state of complement activation, and paroxysms resulting in abrupt hemolysis are frequently triggered by surgery, infection, and/or inflammation. During these episodes, patients with classic PNH, often show dark urine due to marked hemoglobinuria.

Classic PNH is characterized by florid intravascular hemolysis as revealed by a markedly elevated LDH, reduced haptoglobins, and unconjugated hyperbilirubinemia, and increased reticulocytes due to a compensatory BM response, and most patients experience constitutional symptoms such as lethargy and asthenia. In contrast, in patients with PNH in the setting of another BM failure syndrome (AA-PNH and MDS-PNH), BM failure dominates the clinical picture and hemolysis is usually an incidental laboratory finding. Moreover, in patients with classic PNH, anemia is frequent and the leukocyte and platelet counts are usually normal or nearly normal, whereas leukopenia and/or thrombocytopenia invariably accompany PNH in the setting of another BM failure syndrome. Patients with subclinical PNH differ from patients with AA-PNH and MDS-PNH, because, by definition, they have neither clinical nor laboratorial evidence of hemolysis [12].

Thrombosis in PNH patients often occur at unusual locations, such as the intraabdominal visceral veins, with the Budd-Chiari syndrome, caused by hepatic vein thrombosis, being the most dreaded complication [41–43]. The mechanisms involved may include platelet stimulation, activation of the coagulation cascade, inhibition of fibrinolysis, and endothelial activation and dysfunction; these may be caused by complement activation (CD55/CD59 deficiency), toxicity of free hemoglobin, nitric oxide depletion, and absence/reduced expression of GPI-AP with fibrinolytic or anticoagulant properties [41,44,45]. The molecules potentially involved comprise, among others, CD87 (uPAR, urokinase-type plasminogen activator receptor), which binds to urokinase and promotes conversion of plasminogen to plasmin [46,47], heparan sulfate, TFPI (tissue factor pathway inhibitor), a serine protease inhibitor that inhibits tissue factor (TF)-formation and regulates the TF-dependent pathway of blood coagulation, and CD177, which co-localizes with proteinase-3, an enzyme that cleaves the thrombin receptor, thereby decreasing thrombin-mediated platelet activation [48].

Outcomes are affected mainly by disease complications, such as evolution to pancytopenia for classic PNH, occurrence of malignancies for AA-PNH, and thrombosis in both cases [49]. According to the experience of a Spanish center with a series of 56 cases, thrombotic episodes and cancer are the main causes of death among PNH patients, each of them accounting for 8.9% of the deaths [20]. However, it cannot be concluded that PNH predisposes to cancer because, as the authors themselves noted, many of the cancers occurred in PNH patients who had received immunosuppressive treatments.

3. PNH testing and monitoring

In general, testing for PNH is recommended for patients with intravascular hemolysis, acquired BM failure syndromes and thrombosis with unusual features (Table 3) [25,28,50].

A study aimed to evaluate the efficiency of diagnostic screening for PNH by FCM revealed that most of the current medical indications for PNH testing are highly efficient [51]. In this study, 3938 peripheral blood (PB) samples from an identical number of individuals submitted between 2011 and 2014 for PNH screening by FCM at 24 Spanish laboratories plus one reference Brazilian laboratory, were retrospectively evaluated. From the samples screened, 14% were PNH+ and the highest frequency of PNH + cases was observed among patients with hemoglobinuria (48%) and BM failure syndromes (33%), particularly AA (45%) and to a less extent MDS (10%); other efficient clinical indications for PNH screening were hemolytic anemia and unexplained cytopenias, with 19% and 9% of

Table 3
Clinical indications for PNH testing and monitoring.

- Intravascular hemolysis (with or without anemia) as evidenced by increased LDH, decreased/absent haptoglobins, hemoglobinuria and elevated plasma hemoglobin, especially if accompanying by iron-deficiency, abdominal pain, esophageal spasm, thrombosis, and/or neutropenia/thrombocytopenia.
- Bone marrow failure syndromes, including AA and MDS (especially RCUD).
- Thrombosis with unusual features and/or occurring at unusual sites – e.g. hepatic veins/Budd-Chiari syndrome, other intra-abdominal veins (e.g. portal, splenic), cerebral sinuses or dermal veins – especially if accompanied by hemolysis with or without anemia and/or other unexplained cytopenias.
- Regular monitoring of PNH patients, including those receiving eculizumab.
- Regular monitoring of AA-PNH and MDS-PNH patients.

Abbreviations: AA, aplastic anemia; AA-PNH, AA with PNH population; LDH, lactic dehydrogenase; MDS, Myelodysplastic syndrome; MDS-PNH, MDS with PNH population; PNH, Paroxysmal nocturnal hemoglobinuria; RCUD, Refractory cytopenias with unilineage dysplasia.
Based on the recommendations of the *Clinical Cytometry Society* [25] and *British Society for Standards in Haematology* [50], and in the results of the Morado's study [51].

PNH+ samples, respectively. In contrast, only 0.4% of the patients submitted for PNH testing because of unexplained thrombosis in the absence of cytopenias were PNH+. This is in accordance with previous studies revealing that PNH populations are not common in patients with intra-abdominal thrombosis (<1.5% of the cases) [52–54], being even rarer (<0.5% of the cases) in those having unexplained venous thromboembolism [55]. This is because thrombosis has a multifactorial origin and is relatively frequent in the general population, contrasting with the low prevalence of PNH, and indicates that improved screening algorithms for PNH testing are needed for patients presenting with thrombosis, especially those having normal blood cell counts [51].

Once the diagnosis has been established, it has been recommended to quantify the size of the PNH population periodically to monitor disease evolution; the frequency with which the assessment should be made is questionable, although every 6 months or every 12 months is often recommended [25,28,50]. In AA, most studies have shown that the presence of a PNH population is associated with a favorable response to immunosuppressive therapy [37,39,40]. In patients with AA-PNH, the size of the PNH populations may remain stable, decrease or increase, and, in the latter case, patients may develop intravascular hemolysis [56,57]. Thus, AA-PNH and MDS-PNH patients with small PNH populations should be followed because of the risk of developing hemolytic PNH. In accordance, the recent guidelines of the *British Society for Standards in Haematology* recommended that all patients with AA be screened for PNH using FCM, and then reassessed periodically [50].

4. Laboratory tests for PNH

4.1. Specific laboratory tests

4.1.1. Ancillary tests

4.1.1.1. Ham's test. Thomas Ham developed the first assay for PNH diagnosis in 1939, based on the hypothesis that the nocturnal hemolysis observed in patients with PNH was due to decreased blood pH during sleep [58]. In this test, washed RBC are incubated with acidified serum and free hemoglobin liberated by lysis of RBC is quantified by spectrophotometry.

4.1.1.2. Sucrose hemolysis test. Three decades later, Hartmann & Jenkins proposed the "sucrose hemolysis test" for the diagnosis of PNH [59]. In this assay, incubation of blood from PNH patients in hypotonic sucrose solution triggers complement activation and RBC hemolysis.

Although these ancillary tests were used for a long time for the diagnosis of PNH, they are in disuse, mainly because they have a relatively low sensitivity, as compared to FCM [60]. In addition, false positive results are seen in the Ham's test in some hematological disorders such as megaloblastic anemia, spherocytosis and HEMPAS (hereditary erythroblastic multinuclearity with positive acidified serum lysis test, or congenital dyserythropoietic anemia type II). However, it should be noted that HEMPAS is an exceedingly rare disease, and that the Ham's test is negative in HEMPAS when the patient's serum instead of serum of a healthy individual is used. Despite what was said above, the Ham's test still has an important role in PNH diagnosis in many parts of the world where FCM is not easily available.

4.1.2. Flow cytometry

Flow cytometry analysis of PB samples has been applied for the diagnosis of PNH since the mid-eighties. The first FCM tests described were based on the use of unconjugated polyclonal or monoclonal anti-CD55 (DAF) antibodies and indirect fluorescence techniques to identify PNH populations among RBC, white blood cells (WBC) and platelets [61,62]. Sonly thereafter, monoclonal antibodies (mAbs) specific for many other GPI-AP were developed and fluorochrome-conjugated mAbs started progressively to be used [63–70]. In the following years, FCM based tests rapidly proved to be more sensitive and more specific than the ancillary tests. The use of multiparametric FCM subsequently improved the accuracy for detecting PNH populations, and in the last two decades FCM has been widely accepted as the "gold standard" for PNH diagnosis [12,25,71]. The normal patterns of expression of GPI-AP in different subsets of PB cells and in the maturing BM cells have also been studied in detail, to better understand the changes occurring in PNH and to provide reference patterns for PNH diagnosis [72,73].

Different immunophenotypic panels, using mAbs with different specificities and conjugated with diverse fluorochromes have been proposed, with a sensitivity to detect PNH cells at frequencies that ranges from 0.01% (high sensitivity assays) [74] to 1% (low sensitivity assays) [75]. For the diagnosis of classic (hemolytic) PNH in routine clinical practice, a sensitivity of 1% is acceptable; however, detection small PNH populations in patients with BM failure syndromes, such as AA and MDS, needs highly sensitive assays [25,27,74].

Red blood cells, neutrophils and monocytes are the PB cells more frequently tested by FCM. However, FCM can also be used to identify PNH populations in platelets [76], reticulocytes [74,77], eosinophils [78], lymphocytes and other blood cells [72].

The PB of patients with PNH is a mosaic of normal and abnormal cells and the extent to which the GPI-AP deficient clone expands varies widely among patients and among the blood cell types from the same patient [12]. The PNH population size is usually greater in neutrophils and monocytes than in erythrocytes or lymphocytes [79–81], being also usually higher in classic PNH than in subclinical PNH and in PNH in the setting of another BM disorder [34,82]. The International PNH Registry contains substantial information about the size of the PNH population in different groups of PNH patients. In the first 1600 patients enrolled, the median size of the PNH population in granulocytes was off 68.1% (ranging from 0.01%, the minimum inclusion criterion for enrollment, to 100%), and, as expected, it was larger in patients with classic PNH than in patients with AA-PNH (83% vs. 35%) [13]. Also, most (76%) of the PNH

patients without other documented BM disease (e.g. AA or MDS) had large PNH populations in granulocytes (>50%) and only a minority (8%) had small populations (<10%). This study also made clear that the size of the PNH population in granulocytes from patients with other BM failure syndromes is highly variable, with 34% of patients with a history of AA having PNH populations of 50% or higher, and 40% of these patients having PNH populations less than 10% [13].

The degree of GPI-AP deficiency also varies, especially in RBC, being possible to distinguish normal (type I) cells from partially deficient (type II) and completely deficient (type III) PNH cells. Most patients have only type I and type III cells; some patients have type I, type II and type III cells (the second most common phenotype); and a few patients have only type I and type II cells [83]. Type II PNH cells may be observed in RBC (more frequent), WBC or both indicating variable lineage involvement, although the reasons for that are poorly understood [80]. However, sometimes is very difficult to separate type III from type II and type II from type I (normal) cells, as stated by Illingworth et al., who have illustrated these aspects with case studies [32].

4.1.2.1. Red blood cells. CD55 and/or CD59 expression on the RBC was initially used as the only FCM test for PNH. However, RBC analysis may underestimate the size of the PNH population as the pathological RBC are destroyed by hemolysis; in addition, it can be undervalued in transfused patients. Therefore, RBC analysis alone is not recommended (and is not enough) for PNH diagnosis.

CD59 is a better marker for the identification of PNH-clones among RBC, as it stains brightly than CD55; in addition, anti-CD59 mAbs are needed to discriminate type II and type III RBC from normal (type I) RBC [83,84]. A problem, however, is that the available clones of anti-CD59 mAbs differ on their performance for PNH testing, even when using the same fluorochrome; in general, PE conjugated anti-CD59 clones MEM43 or OV9A2 are preferred because of giving the best separation of the HPN RBC populations, whereas other PE conjugated anti-CD59 clones (e.g. p282 and 1F5) do not perform so well [27,31,32,74].

Other GPI-AP expressed on RBC, such as CD58 (LFA-3, lymphocyte function associated antigen), have also been tested, but there is a limited experience in their use in PNH diagnosis [63,70,77]. In accordance, two ancillary studies including a limited number of patients, based on the use of unconjugated mAbs and indirect immunofluorescence techniques, revealed that RBC from PNH patients were CD58 deficient [63,70] and that anti-CD58 normally bounds to deficient WBC and platelets [70], showing the LFA-3 molecule is expressed as a transmembrane protein in these cells. A more recent study, from Höchsmann et al., 2011, included 1296 FCM results from 803 PNH patients examined at diagnosis or during follow-up [77]. In this study, the authors recommended to use anti-CD58 and anti-CD59 for RBC and reticulocyte PNH testing based on the results they obtained with single staining with anti-CD58-PE (clone AICD58, Immunotech) or CD59-PE (clone p282(H19), BD Pharmingen) together with the RNA dye thiazol orange. They found a highly significant correlation between the markers CD58 and CD59 on reticulocytes and RBC; however, the superiority of anti-CD58 over anti-CD59, or of using anti-CD59 and anti-CD58 instead of only anti-CD59 was not investigated [77]. As there are no data indicating that testing for CD58 provides any advantage over CD59 (or CD55), the CD58 (LFA-3) molecule was not considered for using in PNH testing in the guideline manuscripts [25,27,30–32].

The use of forward scatter (FSC) and side scatter (SSC) amplification in log mode to establish an acquisition/analysis gate for RBC is a common practice, and this gating strategy is considered adequate for low sensitivity assays [71,85]. Gating strategies using anti-glycophorin-A (CD235a) mAbs are needed for high sensitivity assays, although anti-CD235a induced RBC agglutination is a major technical problem, and an appropriate antibody titration is mandatory [71,85,86]. Vortex procedures during the incubation period may help to minimize RBC agglutination and the choice of the fluorochrome may also be relevant. In general, FITC-conjugated anti-CD235a gives lower RBC agglutination as compared to PE-conjugated CD235a [87], and PE-conjugated anti-CD59 gives brightly CD59 expression as compared to FITC-conjugated anti-CD59; thus, when using the FL1 and FL2 channels, the best combination is anti-CD235-FITC plus anti-CD59-PE [25,27].

PNH populations are frequently lower in RBC than in WBC, and low sensitivity single staining FCM assays may fail to detect PNH clones in RBC specially in AA-PNH and MDS-PNH, where PNH clones are usually minor [87]. In addition, there are situations in which an incorrect diagnosis of PNH could be done if only RBC are studied. For example, very rare congenital deficiencies of CD55 (Inab phenotype) [88–90] or CD59 [91,92] have been described. Moreover, CD55 and/or CD59 deficient RBC have been observed in patients with autoimmune diseases [93,94]. For these reasons, it has long been assumed that testing only for CD55 and/or CD59 expression on RBC is not enough, and at least two cell lineages (RBC and WBC) must be studied to aid the clinical diagnosis of PNH [25].

According to the UK NEQAS survey performed in 2013, based on an electronic questionnaire to which 105 out of 173 centers participating in the UK NEQAS LI PNH EQA program responded, anti-CD235a is the mAb most commonly used for RBC gating (74% of the centers), and the mAb most frequently used to detect GPI-deficient RBC is anti-CD59, either alone (76%) or in combination with anti-CD55 (23%) [29].

Dual staining with CD235a and CD59 has proved to be capable of detecting PNH RBC at 0.01% sensitivity, and to discriminate type III from type II + III PNH [74]. However, it is often difficult to separate these subsets, especially in patients with large PNH clones receiving RBC transfusions, because old (transfused) RBC often have low levels of CD59 expression. Very recently, Sutherland et al. have shown that using a 3-color RBC staining consisting of allophycocyanin (APC)-conjugated anti-CD71 (transferrin receptor, expressed in immature RBC)/CD235a-FITC/CD59-PE instead of the 2-color CD235a-FITC/CD59-PE allows to discriminate immature (CD71 negative) from mature (CD71 positive) RBC, improving both quantitative and qualitative assessments of the erythroid component in PNH [95].

We have used for a long time a 4-color tube (anti-CD59-FITC/anti-CD55-PE/anti-CD235a-APC/anti-CD45-KO) for an accurate selection of the RBC ($CD45^-$and CD235a+), for the identification of type I ($CD59^+$), type II (CD59 + low) and type III ($CD59^-$) RBC populations, and for the quantification of PNH (type II + type III) RBC (Fig. 1), with high sensitivity (0.01%). However, we subsequently changed to a 3-color combination (anti-CD59-PE, anti-CD235a-APC and anti-CD45-KO), with better discrimination of type II RBC

populations, at lower cost, confirming previous observations on the lack of utility of CD55, and the advantages of using PE-conjugated anti-CD59 in high sensitivity RBC PNH assays [25,27,31,32,74]. We are now considering omitting also the anti-CD45 mAb we have been using in our panel to exclude WBC from RBC analysis because this reagent is not recommended for use in the PNH RBC assay in any guidelines published [25,27,31,32,74]. In addition, adding anti-CD71 to the panel will probably contribute for a better discrimination and quantification of the PNH RBC clone, according to the recent evidences [95].

Virtually all the issues relating to good FCM assay design for PNH testing in RBC can be found in the last ICCS/ESCCA Consensus Guidelines [31,32], and other previous publications [27,74]. All together, these studies cover reagent selection (e.g. specificity, fluorochromes and clones of the mAbs) and experimental procedures (e.g., staining and washing), at the same time they provide information

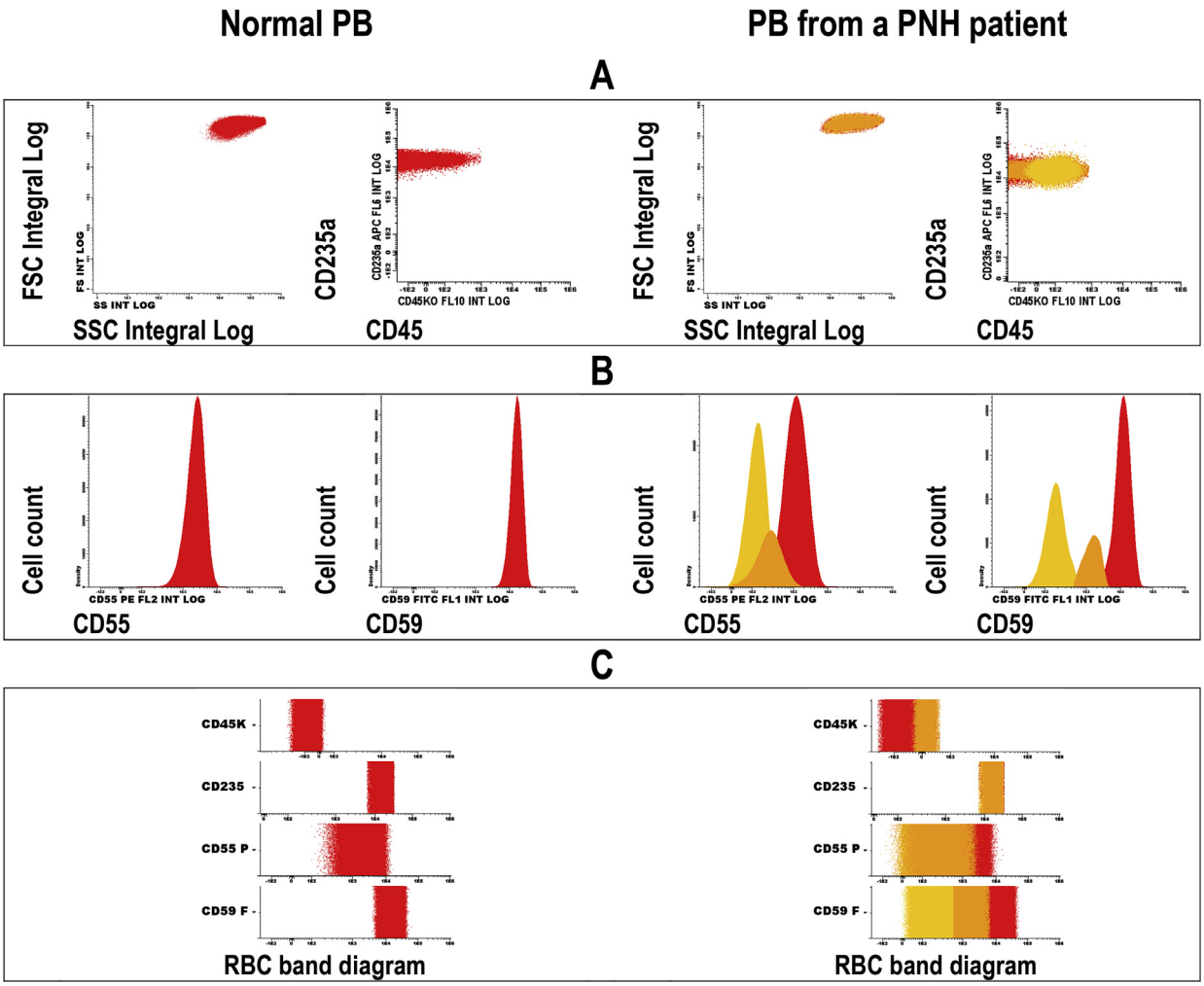
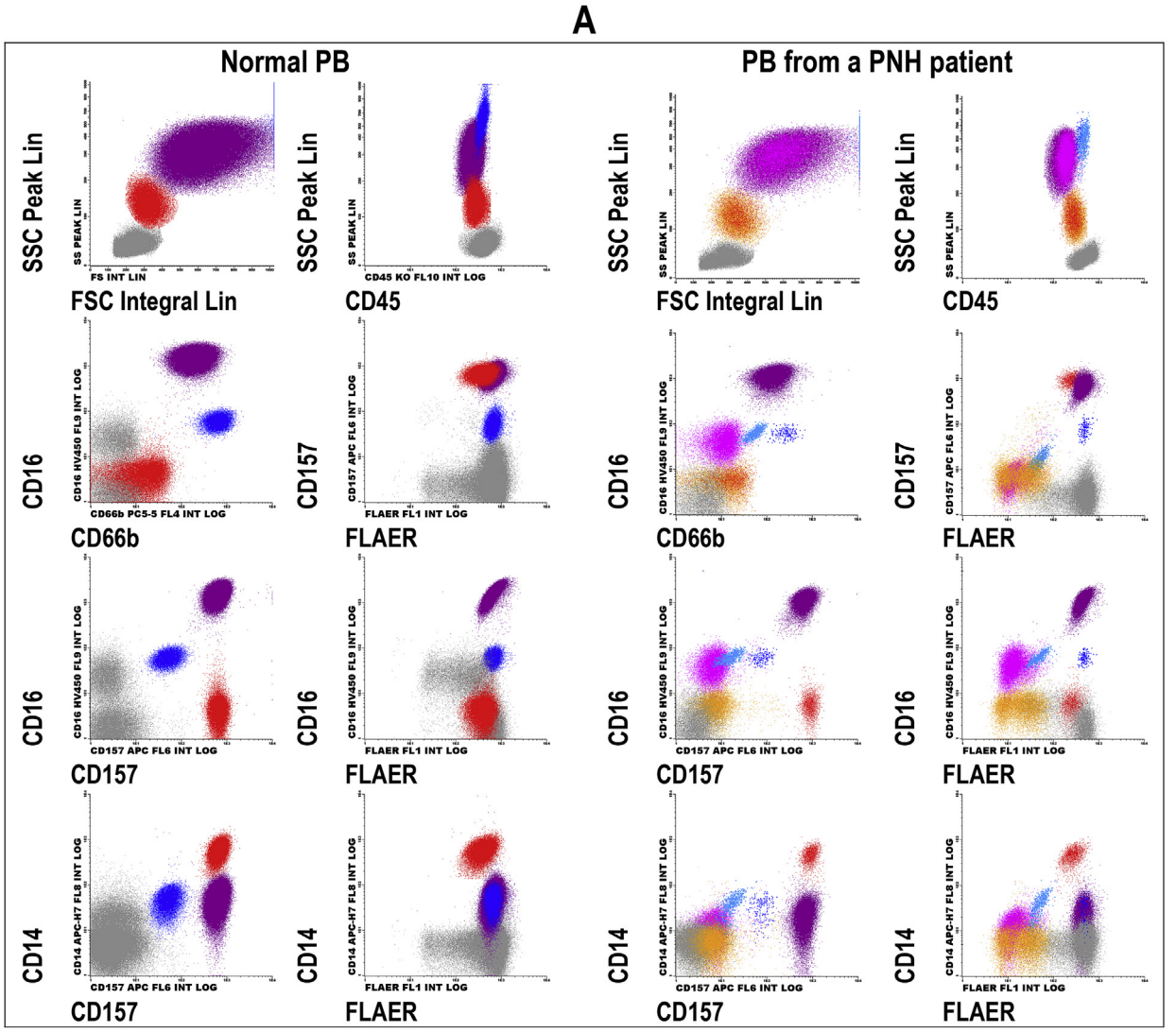


Fig. 1. Flow cytometry studies for the identification and quantification of GPI-AP deficient RBC.
Bivariate dot plots (Panel A) and band diagrams (Panel B) obtained from FCM analysis of the peripheral blood RBC of a healthy individual and of a patient with classic PNH, using the Infinicyt ™ software, version 1.8.0 (Cytognos, Salamanca, Spain).
Please note that the normal PB sample has only type I RBC (red dots, normal CD59 expression, 100%), whereas in the PB sample from this PNH patient, type I RBC (red dots, normal CD59 expression, 48.2%) coexist with type II (orange dots, partial CD59 deficiency, 17.4%) and type III (yellow dots, complete CD59 deficiency, 34.4%) RBC. Red blood cells were stained with the following combinations of mAbs: anti-CD59-FITC (clone MEM-43; Invitrogen), anti-CD55-PE (clone IA10; Becton Dickinson), anti-CD235a-APC (clone GA-R2/HIR2; BD Biosciences), and anti-CD45-KO (clone J.33; Immunotech). RBC were washed twice after staining. FCM was performed using a Navios™ flow cytometer (Beckman Coulter). The minimum number of RBC events acquired per tube was of 250,000; whenever possible, a higher number of events was acquired, up to 1,000,000. All the events acquired were recorded and stored as listmode files (.lmd) and then converted into flow cytometry standard (.fcs) 3.0 files for data analysis. FSC and SSC, represented as FSC and SSC integral, were captured on a logarithmic scale; for fluorescence parameters, a logarithmic amplification was also used. RBC were selected based on their light scatter profile, on the absence of CD45 expression, and positivity for CD235a, and then analyzed for the expression of CD55 and CD59.
Abbreviations: APC, Allophycocyanin; FCM, Flow Cytometry; FITC, Fluorescein Isothiocyanate; FSC, Forward Scatter; GPI-AP, Glycosylphosphatidylinositol-anchored proteins; KO, Krome orange; PB, peripheral blood; PE, Phycoerythrin; PNH, Paroxysmal nocturnal hemoglobinuria; RBC, Red blood cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



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useful for assay validation and data analysis.

4.1.2.2. White blood cells. WBC analysis by FCM has advantages in PNH population detection and size assessment. Neutrophils and monocytes are most commonly used for PNH analysis and are preferred over lymphocytes, which have a more variable expression of GPI-AP; more important, lymphocytes are long-lasting cells and do not, therefore, reflect disease-extent as accurately as the cells having shorter half-lives such as monocytes and granulocytes [25].

Gating based only on FSC/SSC or CD45/SSC dot-plots does not guarantee the purity of the selected leukocyte population. For instance, neutrophils and monocytes may be contaminated with eosinophils and dendritic cells, respectively. As so, mAbs against lineage-specific integral membrane proteins have been used for lineage identification (e.g., CD15 and/or CD33 for neutrophils; CD33 and/or CD64 for monocytes). This is particularly important for high sensitivity assays.

Most studies have shown that CD15 and CD64 are more effective than CD33 for selecting neutrophils and monocytes, respectively. Consequently, current and previous guidelines have recommended the use of anti-CD15 (for neutrophils) and CD64 (for monocytes) in WBC PNH staining [27,31]. However, it should be mentioned that most anti-CD15 mAbs are of the IgM subclass, and are they are known for a long time to induce neutrophil agglutination, needing for a careful titration; in addition, the available clones differ on their performance to discriminate neutrophils from monocytes [27,31].

Based on the assays developed for RBC, anti-CD55 and anti-CD59 were the mAbs initially used for detection of PNH-WBC populations [61,62,69]. In general, CD55 proved to be better than CD59 for WBC analysis; however, different types of WBC have variable expression of these molecules, which makes the interpretation of results problematic [72]. As so, neither CD55 nor CD59 is recommended for WBC analysis over better reagents available nowadays [27,31].

To improve diagnostic criteria for PNH, the normal patterns of expression of GPI-AP on different subsets of PB cells were previously characterized in detail [72]. GPI-AP that have been preferred to identify PNH-neutrophils include CD16, CD24, CD66b and CD157; and PNH-monocytes have been frequently detected based on the analysis of CD14, CD48, and CD157 expression.

Similarly, there are situations in which an incorrect diagnosis of PNH could be done if only one mAb is used to identify PNH populations in WBC. For example, genetic deficiency of CD16 determining the presence of CD16 negative neutrophils in the PB may occur, although this is a rare condition [96], and patients with MDS may have abnormal expression of some GPI-AP (e.g., low levels of CD16 expression in neutrophils) [34]. For these reasons, it has been assumed for many years that the loss of at least two GPI-linked antigens per lineage assessed in WBC must be established before a 'PNH phenotype" can be assigned in the WBCs.

Regarding the CD16 receptor, attention should be paid to the specificity of the mAb used. CD16, the low affinity Fc gamma receptor III for IgG (FcγRIII), is one of the few receptors known to exist natively as a transmembrane protein (CD16a, FcγRIIIA, 50–65 kDa) and as a GPI-anchored protein (CD16b, FcγRIIIB, 48 kDa) [97–99]. CD16a is expressed in most NK cells, some T cell, dendritic cell, and monocyte subsets, and macrophages. It requires association of the gamma subunit of Fc epsilon RI (FcεRI) or the zeta subunit of the TCR-CD3 complex for cell surface expression and is responsible for antibody-dependent cell cytotoxicity. CD16b, the GPI-anchored form, is expressed mainly in neutrophils, is involved in neutrophil activation and induction of an adhesive phenotype and has two polymorphisms (NA-1 and NA-2). GPI-deficient cells from PNH patients lack CD16b, but not CD16a, expression. Most of the mAbs that have been used for PNH screening react both with CD16a and CD16b, for example, the clones 3G8 [77,81,100,101], B73.1 [52], and NKP15 [79]. These anti-CD16 mAbs have been used by several groups for PNH testing in WBC assays [52,77,81,101], although anti-CD16 was not included in the WBC panels recommended for PNH testing in the most recent guidelines [25,27,31,32,74].

The development of fluorochrome-conjugated mutant aerolysin toxin (FLAER) [102], which binds to the GPI-anchor, but does not trigger cell lysis, allowed for the implementation of a new FCM-based strategy to identify PNH cells [103]. The FLAER assay is used only

Fig. 2. Flow cytometry studies for the identification and quantification of GPI-AP deficient WBC cells.
Bivariate dot plots (Panel A) and band diagrams (Panel B) obtained by FCM analysis of the peripheral blood WBC of a healthy individual and of a patient with classic PNH, using the Infinicyt ™ software, version 1.8.0 (Cytognos, Salamanca, Spain).
Please note that in the normal PB sample neutrophils are FLAER+, CD157+, $CD16^{+}$ and CD66b+ (purple dots) and monocytes are FLAER+, CD157+ and $CD14^{+}$ (red dots). In contrast, in the PB from the PNH patient, normal neutrophils (purple dots; 74.4%) coexist with PNH neutrophils (pink dots; FLAER-, CD157-, $CD16^{-}$, CD66b-; 25.6%) and normal monocytes (red dots; 16.4%) coexist with PNH monocytes (orange dots; FLAER-, CD157-, and $CD14^{-}$; 83.6%). In addition, normal eosinophils (dark blue dots; 70.3%) and PNH eosinophils (light blue dots; 29.7%) also coexist in the patient' PB sample, whereas lymphocytes (gray dots) are normal in both samples. Cell staining was done using a stain-lyse-and-then-wash method, and the BD FACS™ Lysing Solution (Becton Dickinson), according to the instructions of the manufacturer. Peripheral blood cells were stained with FLAER (Alexa 488; Cerdarlane) and the following combination of mAbs: anti-CD64-PE (clone 22; Immunotech), anti-CD66b-PC5.5 (clone G10F5; Biolegend), anti-CD10-PC7 (clone ALB1; Immunotech), anti-CD157-APC (clone SY11B5; eBiosciences), anti-CD14-APC-H7 (clone MOP9; BD Pharmingen), anti-CD16-V450 (clone 3G8; BD Horizon), and anti-CD45-KO (clone J.33; Immunotech). FCM was performed using a Navios™ flow cytometer (Beckman Coulter). The minimum number of neutrophils and monocyte events acquired per tube was of 250,000; whenever possible, higher numbers of events were acquired, up to 500,000 events for each cell population. All the events acquired were recorded and stored as listmode files (.lmd) and then converted into flow cytometry standard (.fcs) 3.0 files for data analysis. FSC and SSC, represented as FSC integral and SSC peak, were captured on a linear scale; for fluorescence parameters, a logarithmic amplification was used. The WBC populations were selected based on their light scatter profile and on the expression of CD45 and non-GPI-AP (e.g. CD10 and CD64, for neutrophils and monocytes, respectively), and then analyzed for the expression of the GPI-AP mentioned above. **Abbreviations**: APC, Allophycocyanin; FCM, Flow Cytometry; FITC, Fluorescein Isothiocyanate; FSC, Forward Scatter; GPI-AP, Glycosylphosphatidylinositol-anchored proteins; KO, Krome orange; PB, peripheral blood; PE, Phycoerythrin; PC5.5, PE-Cyanine 5.5; PC7, PE-Cyanine 7; PNH, Paroxysmal nocturnal hemoglobinuria; WBC, White blood cells; V450, Violet 450. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

in WBC studies, as RBC express glycophorin A, a non-GPI-AP that can weekly bind aerolysin [103].

When FLAER is combined with mAbs specific for GPI-AP normally expressed on neutrophils (e.g. CD16, CD24, CD66b, CD157) or monocytes (e.g. CD14, CD48, CD157), PNH populations representing as little as 0.01% can be detected, providing that enough cells are acquired [86,87,101,104,105]. According to the UK-NEQAS survey mentioned above, the most commonly used reagents to detect GPI-deficient WBC were FLAER (86%) and anti-CD24 (71%) for granulocytes, and FLAER (63%) and anti-CD14 (81%) for monocytes [29], which were the reagents recommended at the time the survey was implemented (2013) [25,27], and still continue to be suggested by the current guidelines [31].

One of the best combinations described for WBC relies on the use of CD45, CD15, CD64, FLAER and CD157 for WBC, as it allows to detect, in a 5-color tube, PNH populations in neutrophils and monocytes, at a level of 0.01% and 0.04% sensitivity, respectively [74, 105]. The 8-color tube that has been used for PNH screening in WBC in our Laboratory, which combines non-GPI-linked (CD10, CD45, CD64) with FLAER and GPI-AP (CD14, CD16, CD66b, CD157) also allows to detect PNH populations at the 0.01% level (Fig. 2).

In our experience, anti-CD64 allows for a more distinct separation of the monocyte cluster than anti-CD33, as previously stated by other authors [106]. The main reason we use anti-CD10 instead of anti-CD15 (currently recommended) for neutrophil selection is because, as mentioned before, anti-CD15 mAbs causes neutrophil aggregation, requiring a careful antibody titration for each batch of mAb used [27,31]. As anti-CD10 mAbs were not tested in the consensus documents, further studies are necessary to compare the performance of anti-CD15 and anti-CD10 mAbs for neutrophil gating in PNH studies. Among GPI-AP, CD16 and CD14 allow for very good discrimination of PNH and non-PNH populations in neutrophils and monocytes, respectively, and CD157 permits for the same, in both cell populations. We have no obvious reason to use anti-CD16 instead of the recommended anti-CD24 for identifying GPI-AP deficient neutrophils, except that we have been using anti-CD16 (3G8) for many years with very good results and so, till the moment, we did not feel the need of changing.

For routine high sensitivity PNH testing and taking in account economic reasons, in our hands FLAER and anti-CD66b can be dispensed without losing sensitivity and specificity for PNH diagnosis, and a 6-color combination of anti-CD64/CD10/CD157/CD14/CD16/CD45 represents a good 6-color choice for testing simultaneously neutrophils and monocytes. If decided to evaluate only neutrophils, a 4-color combination is also acceptable (anti-CD10/CD157/CD16/CD45). FLAER may be used instead of CD157 in both cases, with similar results. We do not recommend dispensing the use of anti-CD45 because we consider it to be essential for the appropriate leukocyte selection and adequate quantification of the PNH population, especially in samples with severe leukopenia or with RBC resistant to lysis, as it frequently occur in patients with AA or MDS. In fact, the absence of CD45 expression is very useful for removing debris, platelets and non-lysed RBC.

As mentioned for RBC, several publications and consensus guidelines had covered all aspects of reagent selection, for high-sensitivity detection of PNH neutrophils and monocytes, including not only the specificities of the mAbs, clones and fluorochromes, but also the best combinations [27,32,38,74]. In addition, specific reagent sets were tested across Beckman and BD Biosciences platforms [107,108].

4.1.2.3. Number of events to be acquired. To obtain a well delimited cluster of PNH cells, a minimum of 50 events is usually necessary. Thus, the minimum number of cells to be acquired for a given cell population (e.g. RBC, neutrophils, monocytes) depends on the level of sensitivity desired: 5.000 events for low sensitivity (1.0%), 50.000 events for intermediate sensitivity (0.1%) and 500.000 events for high sensitivity (0.01%) assays. The recently published ICCS/ESCCA consensus guidelines recommended to acquire at least 500.000 events for each cell population analyzed in high sensitivity assays (lower limit of quantification 0.01%, 50 GPI-deficient cells) [32]. Using the currently recommended staining protocols, these numbers are always attainable for RBC and usually achievable for neutrophils, but rarely for monocytes. If there are doubts about the identification of PNH populations, more events should be acquired on the cytometer, for which it may be necessary to duplicate the stainings, in order to have enough cells.

4.1.2.4. Instrument settings, quality control and Proficiency Testing. Assuring proper instrument setup and calibration and appropriate technical procedures for cell staining, acquisition, and data analysis, having adequate internal quality controls and participating in External Quality Assessment (EQA) / Proficiency Testing (PT) (EQA/PT) programs is essential to provide precise and accurate results in PNH testing by FCM. These matters had been addressed in detail in the recently updated 2nd and 4th-parts ICCS/ESCCA consensus documents [31,33].

Concerning the instrument settings for multiparametric cytometry, the *EuroFlow Consortium* has established standardized operating procedures (SOP) [109] and it offers an EQA program using a predefined reagents' panel to test the PB cells of local healthy donors [110]. Although the Euroflow SOP and EQA are not specific for PNH, they allow for monitoring the whole FCM process (e.g., cytometer settings, sample preparation and acquisition, etc.). In addition, consensus guidelines for PNH testing by FCM are available since 2010 [25,26], and specific recommendations for the high-sensitivity PNH assays were published in 2012 [27] and in 2018 [30–33]. These comprise orientations about when testing for PNH and how to perform PNH testing, including selection of mAbs, cell staining procedures, conditions for sample acquisition and data analysis strategies, as well as how to interpret and to report the results obtained, among others.

In respect to the internal quality control, in virtually all samples tested, whether they contain PNH populations or not, normal leukocytes present in the sample should confirm the reactivity of the mAbs/reagents used in the test. In addition, a normal PB sample may be studied in parallel to ensure that under assay conditions 100% of normal cells express the antigens tested. Unstained controls are usually not needed because normal and GPI-deficient cells are typically clearly separated.

Finally, regarding the EQA specific for PNH, the UK-NEQAS LI, an internationally ISO 17043 accredited EQA/PT provider in clinical FCM, has a PNH program for both RBC and WBC (UK NEQAS LI PNH program), in which more than 150 centers from more than 30

countries are participating [29]. In addition, the *Iberian Society for Cytometry* has an EQA scheme for PNH screening based on the analysis of FCM data files, in which several Spanish and Portuguese centers are taking part [51].

Following the current recommendations and participating in the above mentioned EQA/PT programs made possible to perform PNH testing by FCM according to the good laboratory practices.

4.1.2.5. Flow cytometry report and data interpretation. Key information to be included in the FCM report comprises: the level of sensitivity of the assay performed (1%, 0.1%, 0.01%); the specificity of the mAbs used (for cell gating and for identification of GPI deficient cells); detection of a PNH population (yes or no) at least in 2 blood cell populations (usually RBC + neutrophils or neutrophils + monocytes); the total size of the PNH population in each of the cell populations analyzed (neutrophils, monocytes and RBC); the percentages of type II and type III cells, especially in RBC, based on CD59 expression; comparison with previous studies from the same patient (if available); and, if possible, FCM graphs (histograms and/or dot-plots) should be provided [25,32].

In classic PNH, the diagnosis is usually not problematic. However, the way of reporting small PNH populations in patients with BM failure syndromes, such as the AA-PNH and MDS-PNH, is debated, as its significance remains controversial. The *International Clinical Cytometry Society* suggested the following terminology of reporting PNH populations: PNH population >1%: "PNH clone"; PNH population between 0.1% and 1%: "minor population of PNH cells" or "minor PNH clone"; PNH population <0.1%: "rare cells with GPI deficiency" or "rare cells with PNH phenotype" [25,32].

As mentioned above, in patients with BM failure syndromes PNH population sizes may remain stable, decrease or increase over time, and patients experiencing an increase in the PNH population may develop intravascular hemolysis. Thus, AA and MDS patients with small PNH populations should be followed because of the risk of developing hemolytic PNH.

4.1.3. Summary

The antigen specificities of the mAbs more frequently used in PNH studies for selecting the different blood cell populations and for identifying PNH-clones are summarized, and the most relevant recommendations concerning the laboratory good practices and technical aspects are specified in Table 4.

FCM studies using FLAER and fluorochrome conjugated mAbs against GPI-AP and non-GPI-AP should be considered the appropriate method for high sensitivity tests to detect minor PNH populations, and they are recommended when studying BM failure syndromes, such as AA and MDS [111–114]. A comparable sensitivity can be achieved with non-FLAER multiparametric FCM assays, providing the appropriated combinations of mAbs are used [111]. However, it should be stated that there is an important difference between harboring small populations of GPI-AP deficient blood cells and having PNH, and that the use of high-sensitivity techniques may lead to unappropriated diagnosis, if the laboratory results are not interpreted in the clinical settings. Finally, it should also be noted that robust criteria for PNH diagnosis must be reconciled with reasonable economics, and that cost/benefit analysis of using large panels of mAbs is still missing.

4.2. Other laboratory tests

Other laboratory tests are recommended in patients suspected of having PNH, to document the existence and severity of the hemolytic anemia and/or the associated cytopenias, as well as to exclude other possible causes for the anemia and other cytopenias. Once the diagnosis of PNH is confirmed, certain exams are suggested with the purpose of documenting organ damage and/or to exclude or confirm disease complications, such as thrombosis (Table 5).

4.2.1. Blood tests

Routine blood tests should include a complete blood cell count with reticulocyte count, to confirm the existence of anemia and other cytopenias and to assess the BM response, as well as biochemical tests to evaluate the existence of intravascular hemolysis. To exclude other causes of hemolytic anemia, such as autoimmune and congenital hemolytic anemia due to membrane, enzymatic or hemoglobin defects, other tests may also be needed in specific cases (Table 5).

Some groups have recommended testing serum erythropoietin (EPO) levels in PNH patients, presumably because low EPO serum levels may be useful for selecting patients for recombinant EPO therapy [28]. In fact, previous studies have shown that recombinant EPO may be appropriately and safely used in the long-term correction of anemia associated with PNH [115], and more recent studies have revealed that the expression of EPO receptor (EPOR) and other growth factor receptors, is significantly higher on normal ($CD59^+$) than in PNH ($CD59^-$) BM $CD34^+$ cells, with the former having higher levels of STAT5 phosphorylation of these receptors than the later [116,117]. However, the need for measurement of EPO in the serum is controversy, as PNH patients usually have increased EPO serum levels [118,119], and, apparently, the response to recombinant EPO is not dependent on the level of endogenous EPO [115].

4.2.2. Bone marrow analysis

Bone marrow studies, which include BM aspirate and biopsy, should be performed if PNH is suspected to be associated with a second BM disorder (e.g., MDS or AA). They are also useful to confirm an aplastic crisis, which may happen in PNH patients, because of folate deficiency or, more rarely, of infection with parvovirus B19, manifesting as worsening of the anemia and reticulocytopenia (Table 5).

Bone marrow cell immunophenotyping has been done in research settings, to better understand the abnormal patterns of expression of GPI-AP observed in maturating hematopoietic cells from PNH patients [79,101,116,117,120], as compared to healthy individuals [73]. However, they have no diagnostic advantage compared to PB studies, and should not be used routinely for PNH diagnosis. This is

mainly because BM cells are at different stages of maturation and therefore have a variable expression of GPI-AP, which makes interpretation much more difficult.

4.2.3. Genetic studies

The biosynthesis of the GPI anchor is a complex pathway that comprises several enzymatic steps and the corresponding enzymes and genes [121]. *PIGA*, one of the genes involved in the very first step of GPI biosynthesis, is the only that maps to the X chromosome (Xp22.2); thereby, one somatic inactivation mutation in *PIGA* would be enough to cause GPI deficiency, whereas in principle at least two such mutations are required in the other (autosomal) genes [122].

Table 4
Laboratory good practices and technical guidelines for PNH testing by flow cytometry.

	Guideline	Example/Suggestion
1	Use adequate samples and proper immunofluorescence techniques	Sample: Peripheral blood, no more than 48 h old (preferentially 24 h). Anticoagulant: EDTA-K3, heparin (preferentially EDTA-K3). WBC: Stain-and-then-fixe/lyse-and-then-wash, direct immunofluorescence. RBC: Stain-and-then-wash/non-fix/non-lyse direct immunofluorescence. (i)
2	Set the desired level of sensitivity, according to the purpose of the study, and define the number of events to be collected	Low sensitivity (1%): At least 5000 events should be collected for each blood cell type (more events when necessary) (ii) High sensitivity (0.01%): At least 500,000 events should be collected for each blood cell type (more events when necessary).
3	Test at least 2 blood cell populations	Neutrophils + RBC or Neutrophils + monocytes or Neutrophils + RBC + monocytes. (iii)
4	Use monoclonal antibodies against CD45 (for WBC) and at least 1 non-GPI-AP lineage specific marker to better identify each cell population	RBC (CD235a) (iv) WBC (CD45) (v) Neutrophils (CD15 > CD10 > CD33) (vi) Monocytes (CD64 > CD33) (vi) Lymphocytes: B (CD19 or CD20), T (CD3), NK (CD3 and CD56) (vi) Platelets (CD41, CD42b or CD61)
5	Use appropriated gating strategies for each blood cell population	RBC: FSC/SSC - > CD235a+ (vii) Neutrophils: SSC/CD45+ - > $CD15^+$, $CD10^+$, $CD33^+$ (vii) Monocytes: SSC/CD45+ - > $CD64^+$, $CD33^+$ (vii) Lymphocytes: SSC/CD45+ - > B: $CD19^+$, $CD20^+$, T: $CD3^+$, NK: $CD3^-CD56^+$ (vii) Platelets: FCS/SSC - > $CD41^+$, CD42b+, $CD61^+$ (vii)
6	Test at least CD59 for RBC and at least 2 anti-GPI-AP or FLAER + 1 anti-GPI-AP for each WBC population analyzed and identify the PNH populations appropriately.	RBC (CD59; other markers: CD55, CD58, CD71) (viii) Neutrophils (FLAER, CD24 ~ CD157 > CD16 and CD66b > CD55 (ix) Eosinophils (FLAER, CD157 > CD55) (ix) Monocytes (FLAER, CD14 ~ CD157 > CD48 > CD55 (ix) Lymphocytes (FLAER, CD48): B (CD24, CD55), T (CD52, CD55), NK (CD52, CD55) (ix) Platelets (FLAER, CD55, CD59) (ix)

Abbreviations: AA, Aplastic anemia; FLAER, fluorochrome-conjugated mutant aerolysin toxin; FSC, forward scatter; GPI-AP, glycosylphosphatidylinositol anchored proteins; MDS, Myelodysplastic syndrome; PNH, Paroxysmal nocturnal hemoglobinuria; RBC, Red blood cells; SSC, side scatter; WBC, White blood cells.

(i) RBC should be washed twice after staining to decrease non-specific binding of antibody and/or remove excess of fluorochrome.

(ii) Low sensitivity assays (1%) are adequate for the diagnosis of classic PNH, but not to detect small PNH populations in patients with BM failure syndromes (MDS, AA); in this case, high sensitivity tests (0.01%) should be used.

(iii) Neutrophils should always be studied; RBC testing in recommended at least in cases with a detectable PNH-clone in WBC; monocyte testing may not be suitable for high sensitivity analysis because of the difficulty in collecting sufficient events; routine analysis for RBC only is not recommended; lymphocyte and platelet testing are not adequate for diagnosis.

(iv) Anti-CD235a staining is used to better select RBC in high sensitivity assays, but this is not mandatory for low sensitivity (1%) assays. Anti-CD235a mAbs are known to cause RBC aggregation, especially if conjugated with phycoerythrin; mAb titration is needed for each batch used.

(v) Anti-CD45 is useful to proper select WBC, especially in blood samples with leukopenia, nucleated RBC or RBC resistant to lysis, and samples with large platelets or platelet aggregates.

(vi) Except for NK cells, for which both CD3 and CD56 needs to be used, one non-GPI-AP marker is enough for each WBC population. According to the current consensus guidelines, anti-CD24 and anti-CD64 are recommended for neutrophils and monocytes, respectively, as they perform better than CD33. In our experience, CD10 also performs well for neutrophils, being particularly useful to exclude immature granulocytes and eosinophils.

(vii) Use SSC, FSC, and CD45 (for WBC), and the selected non-GPI-APs as surface markers to gate each cell population.

(viii) Anti-CD59 is better than anti-CD55 for RBC; in addition, anti-CD59 is needed to discriminate type II (partial deficiency) and type III (complete deficiency) PNH populations from normal (type I) RBC. There is limited experience with anti-CD58. Anti-CD71 is useful to distinguish mature RBC from immature RBC (reticulocytes and nucleated RBC).

(ix) FLAER + 1 GPI-AP or, alternatively, 2 GPI-AP; depending of using FLAER or not, choose one or two markers for each WBC population, respectively. Bivariate dot plots or density plots are more informative than single parameter histograms.

~ Usually performs equally as; > usually performs better than.

Table 5
Other laboratory tests recommend as part of the initial investigation before establishing the diagnosis of PNH and/or for the evaluation of patients with PNH.

Laboratorial test	Purpose
Full PB count	• Search for anemia and other cytopenias (neutropenia, thrombocytopenia).
Reticulocyte count (corrected)	• Evaluate the BM response.
PB film examination	• Search for RBC abnormalities typically found in other hemolytic anemias (e.g. sickled cells). • Search for the presence of dysplastic neutrophils and other blood cell abnormalities associated with MDS.
Serum LDH and haptoglobins, hemoglobinuria and hemosiderinuria	• Document intravascular hemolysis.
Direct Coombs test	• Exclude autoimmune hemolytic anemia.
Osmotic fragility test, Sickle cell screen and G6PD screen, and other tests for the diagnosis of other hemolytic anemias (before diagnosis) *	• Exclude congenital hemolytic anemia due to membrane (e.g. hereditary spherocytosis), enzyme (e.g. G6PD deficiency), or hemoglobin (e.g. Sickle cell anemia) defects. • Exclude cold agglutinin disease (anti-I/cold agglutinin titer).
Serum iron, ferritin, total iron-binding capacity, unbound-iron binding capacity, transferrin saturation, vitamin B12 and folates	• Search for nutritional deficiencies (iron deficiency is common due to iron loss; folate deficiency is also common and may account for aplastic crisis).
Serum creatinine, urea, creatinine clearance	• Assess renal function and identify renal impairment/damage.
Urinalysis (pH, proteinuria, microalbuminuria, urine sediment); Microbiological study of urine.	• Detect concomitant pathological urinary conditions (e.g. urinary stones, infections) that may contribute to the deterioration of renal function.
Serum EPO	• Select patients for treatment with EPO (controversial).
BM aspirate*	• Evaluate cell morphology (e.g. diagnosis of MDS).
BM trephine biopsy*	• Evaluate BM cellularity (e.g. diagnosis of AA or aplastic crisis).

Abbreviations: AA, aplastic anemia; BM, bone marrow; EPO, erythropoietin; G6PD, Glucose-6-phosphate dehydrogenase; LDH, lactic dehydrogenase; MDS, myelodysplastic syndrome; PB, peripheral blood; PNH, Paroxysmal nocturnal hemoglobinuria.

4.2.3.1. PIG-A gene mutations. In 1993, Kinoshita et al. reported that *PIG-A* was the gene responsible for PNH and that defective surface expression of the GPI-AP was caused by somatic mutations in this gene [100]. One year later, Bessler et al. demonstrated that PNH type II and PNH type III RBC from two patients had different PIGA mutations [123]; however, to the best of our knowledge, the molecular bases for different levels of GPI-AP expression in other blood cells is not clear.

Two years later, Rosse & Ware reviewed the molecular bases of PNH and described a total of 84 *PIG-A* mutations that had been identified in 72 patients [124]. These mutations were spread throughout the entire coding region of the *PIG-A* gene, resulting in non-functional PIG-A proteins. Most of them (63%) were deletion or insertion mutations, and of the mutations identified, 33% were single nucleotide deletions and 7% were single nucleotide insertions; missense mutations, non-sense mutations and mutations at the splice site that affected the PIGA mRNA size and/or stability accounted for 21%, 7% and 8% of the mutations, respectively [124]. Subsequently, it was described that genetic defects in the *PIG-A* gene observed in PNH also include large deletions and small duplications [125]. Succeeding studies have confirmed these observations [126] and revealed that most of PNH patients have multiple mutations [127].

Unexpectedly, some studies have shown that *PIG-A* gene mutations are relatively common in normal hematopoiesis in healthy individuals [128,129]. In PNH patients, the mutations occur in the hematopoietic stem cells and generate a clonal population, whereas in healthy individuals they arise in more differentiated hematopoietic progenitor cells and are usually polyclonal [128,129].

4.2.3.2. Mutations in other GPI genes. Deep next-generation sequencing on all exons of known genes of the GPI anchor pathway in PNH patients without somatic *PIGA* mutations allowed for the identification of mutations in *PIGT*, an autosomal gene (20q13) that is essential for attachment of the preassembled GPI anchor to proteins [130]. Although a single mutation in *PIGA* is known to cause GPI-AP deficiency, at least 2 mutations are required in *PIGT* [122]. In addition, rare inherited hypomorphic (rather than null) mutations of *PIG-A*, *PIGM* and other genes involved in the GPI-pathway have been found to cause diverse congenital abnormalities, seizures and thrombosis, but not PNH [131,132]. Because of this complexity, mutation analysis of the genes codifying for proteins involved in the GPI pathway, including *PIG-A*, are not recommended in routine clinical practice and should be used only in research settings.

4.2.3.3. Other somatic mutations. Whole-exome sequencing of paired PNH and non-PNH cell populations obtained from samples taken from PNH patients and targeted deep sequencing have revealed that, in addition to *PIGA* mutations, other genetic events are frequent in PNH [3]. In accordance, somatic mutations in genes known to be involved in the pathogenesis of myeloid neoplasms (e.g. *TET2, SUZ12*, *U2AF1*, and *JAK2*) are frequently found in PNH patients and they often occur in sub-clones within the *PIGA*-mutant population [3]. This indicates that PNH is a complex disorder orchestrated by many genetic alterations, with some of these mutations probably preceding the acquisition of PIGA mutations, while others occurring later [133].

5. Conclusions

The precise diagnosis of PNH is mandatory, as it has clinical implications for prevention and treatment of adverse events in PNH

patients. It is a consensus that the identification of PNH populations by multiparametric FCM is the "gold standard" assay for PNH diagnosis. This should be performed using FLAER and anti-GPI-AP mAbs, combined with mAbs against non-GPI linked integral membrane molecules, and at least two GPI-AP deficiencies on at least two blood cell types should be documented. It is also accepted that low sensitivity FCM assays (1%) are adequate for the diagnosis of classical PNH, but high sensitivity (0.01%) FCM studies are needed for accurate identification of AA-PNH and MDS-PNH cases. However, some discussion persists regarding the best combination of fluorochrome-conjugated mAbs to be used in the study of the different blood cell populations. Once a PNH population is detected, the results should be viewed in the context of the clinical settings and other laboratory findings, in proper classify the disease into the classic PNH, PNH with another BM disorder and subclinical PNH. Patients with PNH should be periodically reassessed for the size of the PNH clone, every 6-12 months as empirically suggested, or before if there are changes in the clinical condition and/or other laboratory exams.

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Declaration of competing interest

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Abbreviations

AA: Aplastic anemia
AA-PNH: Aplastic anemia with PNH population
ADCC: Antibody dependent cell mediated cytotoxicity
APC: Allophycocyanin
BC: Beckman Coulter
BD: Becton Dickinson
BDB: Becton Dickinson Bioscience
BM: Bone marrow
DAF: Decay accelerating factor (CD55)
EPO: Erythropoietin
EPOR: Erythropoietin receptor
EQA: External Quality Assessment
FcγRIII: Low affinity Fc gamma receptor III for IgG (CD16)
FCM: Flow cytometry
FITC: Fluorescein isothiocyanate
FLAER: Fluorochrome-conjugated mutant aerolysin toxin
FSC: Forward scatter
GPI: Glycosylphosphatidylinositol
GPI-AP: Glycosylphosphatidylinositol-anchored proteins
HEMPAS: Hereditary erythroblastic multinuclearity with positive acidified serum lysis test (congenital dyserythropoietic anemia type II)
IOT: Immunotech
KO: Khrome orange
LFA-3: Lymphocyte function-associated antigen 3
mAb: Monoclonal antibody
MAC: Membrane attack complex
MDS: Myelodysplastic syndrome
MDS-PNH: Myelodysplastic syndrome with PNH population
MIRL: Membrane inhibitor of reactive lysis (CD59)
PB: Peripheral blood
PC5.5: PE-Cyanine 5.5
PC7: PE-Cyanine 7
PE: Phycoerythrin
PIG-A: Phosphatidylinositol glycan, class A
PNH: Paroxysmal nocturnal hemoglobinuria
RBC: Red blood cells
SOP: Standardized operating procedures
SSC: Side-scatter
TF: Tissue factor
TFPI: Tissue factor pathway inhibitor
uPAR: Urokinase-type plasminogen activator receptor (CD87)
V450: Violet 450
WBC: White blood cell