

REVIEW

Paroxysmal Nocturnal Hemoglobinuria Testing by Flow Cytometry: Brief Overview for Clinicians

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glycophosphatidylinositol; PIGA*

ABBREVIATIONS:

AA = aplastic anemia;
DAF = decay accelerating factor (CD55
molecule);
FLAER = fluorescent aerolysin;
GPI = glycosylphosphatidylinositol;
MDS = myelodysplastic syndrome;
PIG-A = phosphatidylinositol glycan
subunit A (gene);
PNH = paroxysmal nocturnal
hemoglobinuria;
RBCs = red blood cells;
RCUD = refractory cytopenias with
unilineage dysplasia;
WBCs = white blood cells;
WHO = World Health Organization.

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ABSTRACT

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, acquired hematopoietic stem cell disorder, caused by a somatic mutation in the **phosphatidylinositol glycan (PIG-A) gene class A**. The gene is responsible for the synthesis of glycosylphosphatidylinositol (GPI) anchor that attaches a number of proteins to the cell surface. The mutant gene leads to partial deficiency or absence of all proteins normally linked to the cell membrane by GPI anchor. The primary clinical manifestations of PNH are complement mediated hemolytic anemia, thrombosis in atypical locations and blood cytopenias. Flow cytometry has become the gold standard method for PNH clone detection due to its high sensitivity and specificity and due to the ability to examine multiple GPI-linked proteins on red and white blood cell surface. It is the method of choice for the detection of very small PNH clones in subclinical PNH that often accompanies aplastic anemia and other bone marrow disorders. PNH clone detection traditionally involves the analysis of CD55 and CD59 on red and white blood cells. Other markers such as CD14, CD16, CD24, CD66b and CD157 are suitable to detect GPI-linked proteins in the surface of granulocytes and monocytes. The most useful reagent to assess white blood cell PNH clones is fluorescent aerolysin (FLAER), which is a mutated form of proaerolysin conjugated with a fluorochrome. Its advantage in PNH clone detection is due to the ability to bind directly to the glycan portion of the GPI anchor. Flow cytometry is a sophisticated method and a useful tool for clinical cell analysis. However, PNH is clinically diagnosed and flow cytometric results should always be interpreted with respect to clinical manifestations and other laboratory findings.

INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, acquired, clonal disorder occurring in a hematopoietic stem cell. It is caused by a mutation of the X-linked phosphatidyl-inositol glycan, (PIG-A) gene class A. PIG-A gene is responsible for the first step in the biosynthesis of the glycosylphosphatidylinositol (GPI) anchor, a glycolipid structure that attaches more than 12 different proteins to the cell surface of blood cells, including blood group antigens, adhesion molecules and complement regulatory molecules.¹ PIG-A gene is located on the X chromosome, however, PNH affects males and females almost equally. That is because males have only one X chro-

mosome and females undergo lyonization (inactivation of one X chromosome) in somatic cells, including hematopoietic stem cells.¹ A large spectrum of acquired *PIG-A* mutations have been described, the majority of which results in an unstable and rapidly degraded protein. In most cases, mutations are unique to a patient, suggesting the absence of a mutational hot spot in the gene.¹ The genetic defect results in partial or absolute deficiency of all GPI-linked proteins in a clone of hematopoietic stem cells and consequently in all lineages arising from it. Deficient cells are referred to as “PNH clones”.

PNH manifests with three distinctive clinical features. Complement mediated *hemolytic anemia* is the best described and most clinically significant consequence of GPI-linked protein reduction.² In normal individuals, two GPI-linked proteins protect red blood cells (RBCs) from complement activity. **Decay accelerating factor or CD55** is one of them. CD55 protects RBCs from hemolysis by inhibiting C3 convertases which enzymatically cleave and activate C3. In the absence of CD55, C3 accumulates on the surface of RBCs and causes their destruction by the reticuloendothelial system in the liver and spleen (extravascular hemolysis).³ **Membrane inhibitor of reactive lysis or CD59** is another GPI-linked protein that prevents RBCs from the lytic activity of the complement by blocking the aggregation of C9 into the membrane attack complex. Membrane attack complex is the final stage of complement activation which forms pore in the target cell. The absence of CD59 from the RBC surface leads to intravascular hemolysis which most contributes to the morbidity of the disease.⁴ Many of the symptoms that accompany PNH, such as dysphagia, recurrent abdominal pain, erectile dysfunction, fatigue, and severe lethargy, are due to reduced level of nitric oxide.⁵ Nitric oxide is produced in the endothelium and regulates vascular and smooth muscle tones and limits platelet activation. Free hemoglobin released from RBCs during intravascular hemolysis scavenges circulating nitric oxide, consumption of which causes vasoconstriction and smooth muscle dystonia.

Thrombosis is another complication of PNH and the most common cause of mortality.⁶ The pathogenesis of thrombosis involves a multifactorial mechanism in which platelet activation, free hemoglobin, nitric oxide depletion, impairment of the fibrinolytic system and inflammatory mediators take part. Thrombotic events may occur at any site, although venous thrombosis is more common than arterial. They often arise in atypical locations particularly in cerebral, hepatic (Budd-Chiari syndrome), portal, mesenteric, splenic, renal and pulmonary veins.

Bone marrow failure is the third element that accompanies PNH. It occurs in almost all patients but its severity varies. Aplastic anemia (AA) is the most extreme form.⁷

The International PNH Interest Group proposed a classification scheme for PNH consisting of three main categories: 1) **Classical PNH** includes patients with clinical evidence of intravascular hemolysis or hemolysis but no evidence of

another defined bone marrow abnormality; 2) **PNH in the setting of another specified bone marrow disorder**; patients in this category have clinical and laboratory evidence of PNH and a defined underlying bone marrow abnormality, such as acquired AA or myelodysplastic syndrome (MDS) of refractory cytopenias with unilineage dysplasia (RCUD) type (according to the last WHO classification); 3) **Subclinical PNH** includes patients with another bone marrow disorder (AA, MDS) who have no clinical or laboratory evidence of hemolysis or thrombosis. However in these patients, small PNH clones can be detected in peripheral blood by very sensitive flow cytometric analysis.⁸ The close relation of acquired AA and PNH is well documented in the literature, but the exact pathophysiologic mechanism that links the two disorders remains unclear. AA and PNH are bone marrow disorders in which hematopoietic stem cell damage stands as common denominator; in case of AA by an autoimmune attack and in PNH by a *PIG-A* gene mutation.⁹ AA may precede PNH diagnosis or less frequently may arise in the setting of preexisting PNH. More than 50% of AA patients demonstrate PNH clones at diagnosis.^{10,11} The clinical significance and the prognostic value of these clones remain controversial. These clones are often very small and they may increase or decrease over time. There are cases in which PNH emerges after immunosuppressive therapy for AA.^{10,12}

Rarely do patients with MDS reveal classic PNH. The fact is explained by the different cells in which *PIG-A* mutation occurs. In acquired AA, *PIG-A* mutation occurs in hematopoietic stem cell that can self-renew and give rise to all hematopoietic cell lineages. In MDS the mutation arises in colony forming cells which lack self-renewing ability and are able to survive only for three to four months.¹³

INDICATIONS FOR PNH TESTING

PNH is a rare disorder that can present with a wide range of clinical signs and symptoms of which none is pathognomonic. However, some clinical presentations raise suspicion for PNH and need further investigation. Patients presenting with hemoglobinuria should be tested for PNH, as well as patients with Coombs-negative hemolytic anemia, particularly those without characteristic cellular abnormalities, such as sickled cells or schistocytes and no obvious infection that can cause hemolysis. Although thrombosis is a common complication, routine screening of all patients with a thromboembolic event is not recommended unless thrombosis appears in unusual sites. Budd-Chiari syndrome, thrombosis of intra-abdominal vessels (inferior vena cava, portal or splenic vein), dermal and cerebral veins are indications for PNH testing, especially if cytopenias and/or intravascular hemolysis coexist. Patients with evidence of bone marrow failure including aplastic or hypoplastic anemia, RCUD and unexplained cytopenia should be investigated for the presence of PNH clones.^{8,14} A summary

of the indications for PNH testing is presented in [Table 1](#).

IMMUNOPHENOTYPING ANALYSIS OF PNH CELLS

Prior to the last two decades, acidified serum lysis test (Ham test) and sucrose lysis test were the classic diagnostic methods to demonstrate the sensitivity of RBCs to complement activation.¹⁵ However, both tests were nonspecific and unable to quantitate the percentage of PNH clones. In 1990, van der Schoot and coworkers demonstrated that the use of monoclonal antibodies to GPI-linked proteins and flow cytometry were suitable in the analysis of granulocyte PNH clones.¹⁶ A year later, Rosse et al and Shichishima et al published their work about the detection of GPI-linked proteins on the surface of RBCs in PNH by flow cytometry.^{17,18} Flow cytometry has become the gold standard method in the diagnosis and monitoring of PNH due to its high sensitivity and specificity and the ability to examine multiple GPI-linked proteins in the surface of multiple blood cell lineages.¹⁴

The preferred specimen for PNH testing by flow cytometry is peripheral blood collected in ethylenediaminetetraacetic acid, heparin or acid citrate dextrose solution. Testing is recommended to be performed preferably within 24-48 h of collection. Bone marrow samples should not be used routinely but only in the setting of a research protocol. This limitation is due to the presence of immature cells of both erythroid and myeloid lineage in which GPI-linked proteins are underexpressed.¹⁴ Flow cytometry is performed by incubating peripheral blood cells with fluorochrome labeled monoclonal antibodies that bind to GPI-linked proteins. Deficiency or absence of GPI-linked proteins from the cell surface indicates the presence

of PNH clones. PNH is a clinical diagnosis that has to be confirmed by flow cytometric examination of peripheral blood in which reduction of at least two GPI-linked proteins on ³two or more cell lineages has to be established^{1,19}.

RED BLOOD CELL ANALYSIS

In high sensitivity flow cytometric assays, recognition of mature RBCs relies on CD235a (glycophorin A), a monoclonal antibody that is used for RBCs acquisition and analysis to further parameters. High sensitivity assays are mandatory for the detection of small PNH clones in patients with AA or RCUD.¹⁴ Regarding RBC evaluation for PNH clones, the first and most frequently studied GPI-linked antigens are CD55 and CD59. However, CD55 is inferior to CD59, as it is less abundantly expressed on RBCs and does not offer the best separation of RBC types.^{14,20} For this reason it is not recommended to be used as a sole reagent. Three different RBC types can be identified and quantified regarding the expression of those two GPI-linked proteins. Type I RBCs are characterized by normal GPI-linked protein expression, type II RBCs show partial deficiency and type III complete absence of GPI-linked proteins.¹⁴ The distribution of these RBC populations show a wide range among patients and the distinction between the various types is not always clear ([Fig. 1](#)). Type III RBCs are the most sensitive to complement-mediated lysis in vivo. It is important to mention that RBC testing alone is not sufficient for PNH clone evaluation because hemolysis and/or hemodilution due to transfusion may underestimate the clone size¹⁴. For these reasons, PNH testing should always be provided on both white leukocytes and RBCs. PNH clones on white blood cells (WBCs) may be detected in the absence of RBC clones, but significant RBC clones are never seen without WBC clones.²⁰ The most common ways of depicting flow cytometric results are histograms, dot and density plots. Single color histogram displays the intensity of the signal recorded on the x axis with the number of cells displaying intensity on the y axis. It is suitable for separation and quantification of type II and type III cells when they represent enough events to form a separate peak.¹⁴ When PNH clones represent a small number of events, dot or density plot are more appropriate to ensure that they represent a distinct population.^{14,20} High sensitivity flow cytometric analysis is capable of detecting small PNH clones represented by 20 clustered type III events in a total of one million RBCs.²⁰

WHITE BLOOD CELL ANALYSIS

Granulocytes and monocytes are the optimal cell type for evaluation of the PNH clone size, especially in heavily pretransfused patients.^{14,20} That is because the life span of

TABLE 1. Indications for PNH testing

<ul style="list-style-type: none"> • Patients with hemoglobinuria
<ul style="list-style-type: none"> • Patients with Coombs negative hemolytic anemia, especially patients without cellular characteristic abnormalities or infection
<ul style="list-style-type: none"> • Patients with venous thrombosis involving unusual sites <ul style="list-style-type: none"> - Budd – Chiari syndrome - Other intra - abdominal veins (e.g. portal, mesenteric, splenic) - Cerebral veins - Dermal veins
<ul style="list-style-type: none"> • Patients with bone marrow failure syndromes (e.g. aplastic or hypoplastic anemia, RCUD) <ul style="list-style-type: none"> - Aplastic or hypoplastic anemia - Refractory cytopenias with unilineage dysplasia - Other cytopenias of unknown origin

PNH = paroxysmal nocturnal hemoglobinuria; RCUD = refractory cytopenias with unilineage dysplasia

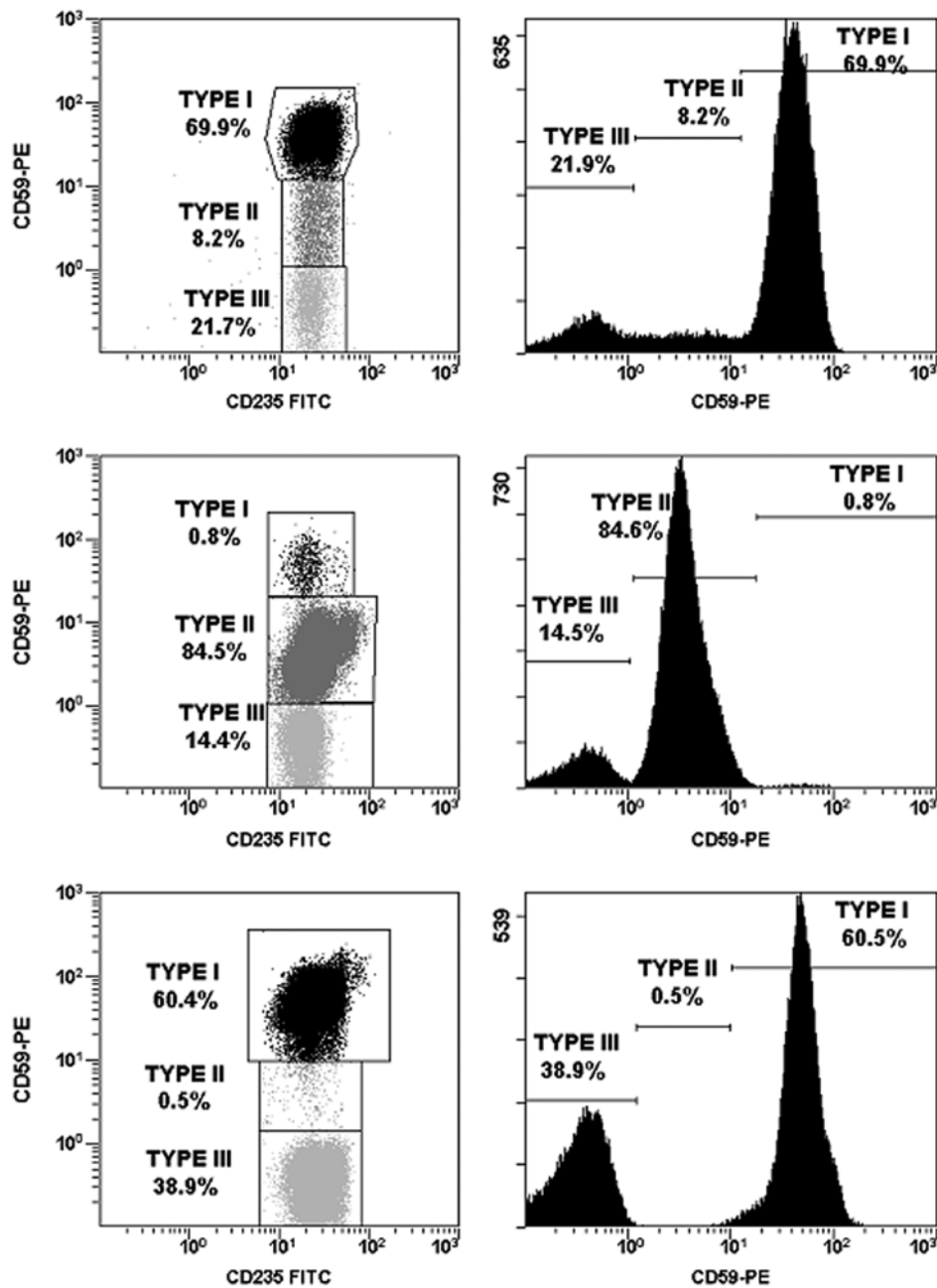


FIGURE 1. High sensitivity flow cytometric evaluation of RBC PNH clones. CD59 expression on RBCs in three cases with different percentages of PNH clones shown on dot plots (left column) and on histograms (right column). The three different PNH types are shown in black (type I), grey (type II) and light grey (type III). The RBCs are gated by CD235a (not shown). PNH = paroxysmal nocturnal hemoglobinuria; RBCs = red blood cells.

PNH granulocytes is normal and the proportion of GPI-linked deficient granulocytes reflects the PNH clone size more accurately.⁸ In contrast, lymphocytes are not suitable for PNH testing because of their prolonged life span and the variability of GPI-linked proteins expressed in their surface. Lymphocytes

arising after the onset of the disorder will be deficient in GPI-linked proteins as granulocytes and monocytes. However, these lymphocytes represent a minority of the entire population. The majority of lymphocytes which are “older”, as they had arisen before gene mutation, will express GPI-linked proteins

normally.^{14,21}

In high sensitivity flow cytometric analysis of granulocytes and monocytes, meticulous gating is assured by using lineage specific non-GPI-linked markers, such as CD15 for granulocytes and CD33 or CD64 for monocytes, combined with light scatter and/or CD45 expression (Fig. 2).¹⁴ Traditionally, CD55 and CD59 are used to detect PNH clones in WBCs as well as in RBCs but these markers are not sensitive enough to assure the detection of small (<1%) PNH clones in WBCs, especially when the investigation concerns AA and RCUD cases. Other markers such as CD14, CD16, CD24, CD66b and CD157 are suitable to detect GPI-linked proteins in the surface of granulocytes and monocytes.^{14,22} However, there are still some limitations, as low expression of a marker does not always mean deficiency or absence of a GPI-linked protein; CD16 may be absent from granulocytes in case of myelodysplasia and CD14 is not expressed in immature monocytes.¹⁴ The most useful reagent to assess the WBC PNH clones is fluorescent aerolysin

(FLAER).²³ FLAER represents a mutated form of proaerolysin secreted by *Aeromonas hydrophila*. It is conjugated with a fluorochrome and the advantage in PNH clone detection is due to the ability to bind directly to the glycan portion of the GPI anchor. The proportions of PNH clones in granulocytes and monocytes are almost equal.¹⁴ FLAER cannot be used to assess PNH clones in RBCs.^{14,20-23} The lower limit of sensitivity in WBC PNH clone detection has not been determined. However, the combination of more than one GPI-linked marker with FLAER in multiparameter flow cytometry and rare event analysis enhances the sensitivity to 0.01%.¹⁴

REPORTING RESULTS AND INTERPRETATION

In the flow cytometry report of PNH evaluation, clinicians get information concerning proportions of PNH clones in

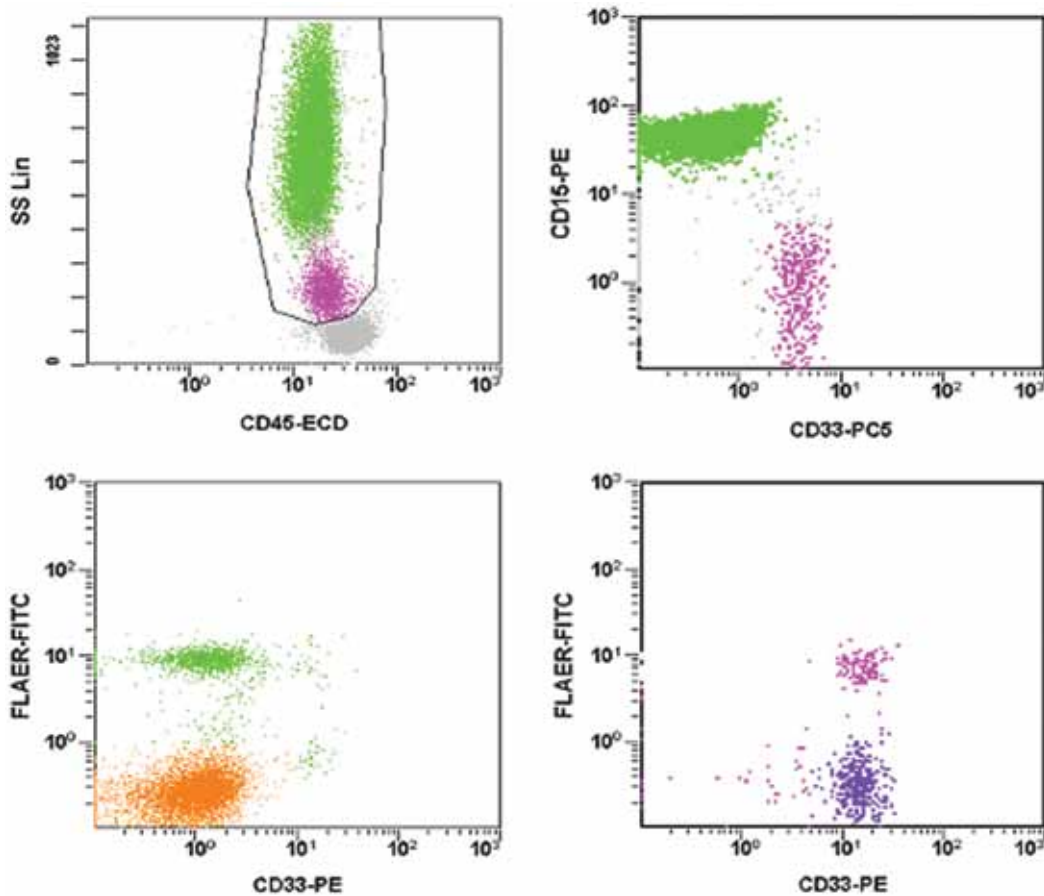


FIGURE 2. High sensitivity flow cytometric evaluation of WBC PNH clones. Granulocytes (green) and monocytes (pink) are identified on the basis of CD45 vs side scatter (top left) and CD33 vs CD15 dot plot (top right). Each of these populations is displayed on a CD33 vs FLAER dot plot (bottom row). PNH granulocytes (orange) are shown in the bottom left plot and PNH monocytes (purple) in the bottom right plot. FLAER = fluorescent aerolysin; PNH = paroxysmal nocturnal hemoglobinuria; WBCs = white blood cells.

RBCs and WBCs (granulocytes and monocytes). As detailed above, PNH RBC clones are identified by the reduced level of GPI-linked proteins CD55 and CD59. The percentage of each of the three RBC types (I, II and III) has to be recorded in the flow cytometric report.¹⁴ The percentage of RBC clones is very often lower than the corresponding WBC clones. As already mentioned, this is due to the shorter life span of RBC clones or to hemodilution caused by transfusion. There is no clear cut off value of RBC PNH clone's size that determines when PNH patients will be symptomatic. However, patients with more than 20% of type III RBCs are more likely to develop intravascular hemolysis, while patients with large type II and absence of significant type III PNH clones may demonstrate less hemolysis.¹⁴ The PNH clone size of granulocytes and monocytes has to be reported also. However, the clone size can be highly variable and it is not a good indicator of PNH severity by itself. In the International PNH Registry, 1610 PNH patients were enrolled. The median granulocyte clone size was 68% of granulocytes. Small granulocyte clones of <10% were detected in 17.4% of patients, clones of 10-49% in 15.3% of patients and large clones of >50% of granulocytes in 51.7% of patients.²⁴ Patients with clone size 50% or more experienced hemoglobinuria, abdominal pain, dysphagia and erectile dysfunction as frequently as patients with clone size less than 10%.²⁴ In addition, there were patients with large PNH clone(s) relatively asymptomatic.⁹ Nonetheless, it has to be emphasized that small PNH clone detection is not equivalent to classical PNH diagnosis.¹⁴ As it has already been mentioned, small PNH clones can be detected in patients with AA and RCUD and even very small clones have been observed in healthy control subjects.^{14,19}

Patients with established PNH diagnosis have to be monitored annually by flow cytometry, regardless of the PNH clone size, as the clone size may increase or decrease over time. PNH clone size monitoring more frequently than once a year is not required.^{1,19}

In **conclusion**, flow cytometry is the method of choice for PNH clone evaluation regarding patient diagnosis and monitoring. Higher sensitivity assays and rare event analysis can accurately detect clones in classical PNH but even very small PNH clones in subclinical PNH. However, PNH is a clinical diagnosis and flow cytometric results should always be interpreted with respect to clinical manifestations and laboratory findings.

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