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Expert Consensus on the Diagnosis and Monitoring of Paroxysmal Nocturnal Hemoglobinuria (PNH): An Algorithmic Approach in an Era of New Treatments

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ABSTRACT

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare hematologic disorder caused by a defect of glycosylphosphatidyl-anchored proteins, leading to an uncontrolled complement-mediated hemolysis. The advent of complement inhibitors in clinical practice radically changed patients' outcomes and survival. Despite this significant progress, numerous unmet needs persist in clinical practice. A group of Italian experts explored the main challenges currently associated with PNH diagnosis and monitoring and reached a consensus on screening, diagnosis, initial work-up, and monitoring. The following core questions were examined: (i) Which patients need to be tested? (ii) Which test to use, and how do we define positivity? (iii) Which workup is requested for patients with a newly detected PNH clone? (iv) Which monitoring in untreated patients? (v) Which monitoring in C-terminal-inh and C-proximal inh? Each specific clinical question was framed according to the GRADE format. Overall, flow-cytometry for PNH detection was recommended in patients presenting with two or more suggestive signs or symptoms and/or with hematologic conditions known to be associated with PNH clones, including DAT-negative hemolysis, idiopathic venous thrombosis (particularly at atypical sites), and primary bone marrow dysfunction (such as aplastic anemia or myelodysplastic syndrome). A high-sensitivity flow cytometry test in neutrophils and erythrocytes should be preferred (lower limit of quantification 0.01%). Baseline assessment of disease activity (i.e., LDH > 1.5×ULN and PNH signs/symptoms), thrombotic risk, bone marrow (BM) features and imaging for organ dysfunction are suggested. Monitoring comprises blood counts and hemolytic markers with frequency depending on anemia severity and symptoms, as well as annual PNH clone retesting, BM reevaluation in case of cytopenias, and constant education about infectious risk and breakthrough hemolysis in patients on treatment.

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1 | Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare hematologic disorder caused by somatic mutations in the X-linked phosphatidylinositol glycan anchor biosynthesis class A (PIGA) gene, leading to a complete or partial deficiency of glycosylphosphatidyl-anchored proteins (GPI-APs). Since the GPI-AP CD55 (decay-accelerating factor for complement) and CD59 (membrane attack complex [MAC] inhibitory factor) are responsible for protecting red blood cells (RBC) from complement-mediated lysis, their absence makes PNH erythrocytes susceptible to intravascular hemolysis and thrombosis. Hemolytic signs of PNH are linked to the expansion of many clones that lack GPI-APs [1]. The clinical manifestations of PNH are hemolytic anemia, thrombophilia, and bone marrow failure [2–5].

In addition to the classic triad of hemolysis, thrombosis, and bone marrow failure, however, patients may present with a complex combination of signs and symptoms (Table S1), including asthenia (80%), dyspnea (64%), hemoglobinuria (62%), abdominal pain (44%), erectile dysfunction (38%), chest pain (33%), and dysphagia (>10%) [6, 7]. These signs and symptoms are not specific and, therefore, may lead to a delay in diagnosis and treatment.

The clinical conditions where it has been suggested to rule out the presence of a PNH clone [8, 9] include two major categories of patients showing two or more signs or symptoms of PNH (Table S1) and with hematologic conditions associated with the presence of a PNH clone (Table S2), particularly Coombs negative hemolytic anemia and bone marrow failure. The synergic effect of signs and symptoms on the prevalence of PNH clones is summarized in Table S3.

Historically, the treatment of PNH was exclusively supportive, and the median survival of patients was 15–20 years, thrombosis being the major cause of death [10]. With the advent of complement inhibitors in clinical practice, the survival of patients with PNH has become close to that of sex- and age-matched controls [11]. Since the first approval of eculizumab for PNH patients, the therapeutic scenario of PNH has been rapidly changing, and several complement inhibitors are currently reimbursed. Two classes of complement inhibitors are available: terminal complement inhibitors, which target C5, and proximal complement inhibitors, which focus on C3 or alternative pathway factor B and D.

Despite such a huge advancement, several unmet needs are recognized in clinical practice, mostly consequent to the rarity of the disease and the limited expertise of several centers in diagnosis and treatment.

A group of Italian experts explored the main challenges currently associated with PNH diagnosis and monitoring and reached a consensus focused on PNH test selection and result interpretation aimed at screening and diagnosis, work-up of newly diagnosed patients, and monitoring of treated ones. A simplified evidence-based process was therefore framed to provide transparent consensus statements.

2 | Methods

An Expert Panel of 9 senior hematologists was invited to join the project. The female gender was appropriately represented (6 out of 9 panelists). The panelists joined from 9 different centers located in the North, Center, and South of the country. Selection was based on prior participation as principal investigators in clinical studies devoted to PNH and being a coauthor of published manuscripts on PNH in the last 5 years.

2.1 | Phase 1. Question List

The panel first met in a virtual event on 16th July 2024. During the meeting, the domain was framed into 3 major clinical actions: (a) diagnosis, (b) workup, and (c) monitoring. Subsequently, the specific clinical questions were framed according to the GRADE format. According to the general Patient (P)-Intervention (I)-Outcome (O) framework, the panelists listed 4 P-I-O questions:

1. Which individuals (P) need to receive testing for PNH (I) to appropriately manage and prevent disease complications (O)?
2. Which tests (I) should be applied to patients (P) when PNH needs to be ruled in/out to get the appropriate diagnostic yield, reproducible results, and clone size in different cell lineages (O)
3. Which workup (I) is requested for patients newly detected as carriers of a PNH clone (P) to assess disease diagnosis and prognosis (O), but also disease activity and those peculiarities (O) prompting specific prevention activities?
4. Which monitoring (I) is necessary in patients harboring a PNH clone but do not receive complement inhibitor therapy (P) to timely detect clone expansion and critical disease activity (O) and therefore timely start target treatment and prevent disease complications (O)?
5. Which monitoring (I) is necessary for PNH patients receiving terminal complement inhibitors or proximal complement inhibitors (P) to assess response (O), customize therapy to the specific patient (O), timely detect hemolytic flares (O), and surveil disease and treatment complications (O)?

2.2 | Phase 2. Evidence Review

A non-systematic evidence search was completed by two major EMBASE queries on 10th September 2024:

a. 'paroxysmal nocturnal hemoglobinuria'/exp. AND sensitivity
b. 'paroxysmal nocturnal hemoglobinuria'/exp. AND monitoring

By the first query, we retrieved 482 records, while 326 records were selected by the second one. The list of records (title, reference, abstract) was provided to all the panelists.

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Each clinical question was assigned to two panelists who oversaw revising the evidence and detailing the outcomes.

2.3 | Phase 3. Consensus

Based on the evidence review and the GRADE Working Group checklist, the panelists proposed practice statements [12].

To label "good practice statements", the GRADE Working Group checklist was applied: (1) Is the statement clear and actionable? (2) Is the message really necessary regarding actual health practice? (3) After consideration of all relevant health outcomes and potential downstream consequences, will implementing the good practice statement result in large net positive consequences? (4) Is collecting and summarizing the evidence a poor use of a guideline panel's limited time and energy? (5) Is there a well-documented, clear, and explicit rationale connecting the indirect evidence? [13].

If the statements did not adhere to all GRADE checklist criteria, the panelists ascertained whether it might be appropriate to assign such a statement to the "background statements" list or to the "research statements" one. In no case was a systematic review of the evidence deemed feasible or relevant.

To better frame the statements devoted to Question n.1, clinical and laboratory hints for suggesting a possible PNH suspicion were ranked by the panelists. First, a list of combined frames was completed. Subsequently, panelists ranked PNH screening as "mandatory" in those individuals showing a high probability of harboring a PNH clone and for whom the drawbacks of missed diagnoses were high. They also ranked PNH testing as "recommended" in specific subgroups for whom either the probability of a PNH clone or the drawbacks of a missed diagnosis were relevant. PNH testing was also considered an "optional" action in those individuals for whom the rate of PNH clones was quite low, and no drawback was universally anticipated.

The proposed statements were voted on by a Nominal Group Technique method during the meeting held in Milan on 4th December 2024. The approved statements required the full agreement of at least 7 panelists, namely > 75%.

The present manuscript adheres to ACCORD standard reporting guidelines [14].

3 | Results

3.1 | Q1. Which Patients Need to be Tested?

This topic is fundamental for determining which signs and symptoms warrant screening for a PNH clone. Identifying the clone and selecting the appropriate patients for testing enables clinicians to manage specific clinical conditions effectively, prevent disease complications, and avoid unnecessary investigations.

Morado et al. [15] studied the frequency of clones at diagnosis in high-risk patients in Spain and Brazil. Out of 3938 cases tested, 563 were positive for a PNH clone: 34% with bone marrow failure, 24% with hemolysis, 8.8% with cytopenia, and 1.5% with thrombosis. Cannizzo et al. [16] found 583 PNH clones out of 3783 cases tested, divided by "reason for testing" as follows: 183 with hemoglobinuria and/or hemolysis, 144 with bone marrow failure, 44 with myelodysplastic syndrome (MDS), 15 with thrombosis at atypical sites, and the rest with unreported diagnosis.

Not all signs and symptoms have the same predictive value in defining the presence of a clinically significant PNH clone, especially when detected singularly. However, when signs and symptoms are combined, the probability of identifying a PNH clone increases [15]. Therefore, some experts [17] suggest searching for PNH in patients with anemia or hemoglobinuria associated with direct antiglobulin test (DAT)-negative hemolysis, idiopathic venous thrombosis, or unexplained cytopenia.

In patients with aplastic anemia (AA), the PNH clone should be monitored during follow-up, even if absent at diagnosis. PNH clone, mostly of medium size, is frequently present in patients with AA and that it may increase and evolve into classical PNH in more than 40% of cases [18–21]. In 131 patients with AA and PNH clone in follow-up, there was no difference in hematological response to immunosuppressive therapy between those with an evolving pattern and those with a stable pattern [22]. By contrast, in 102 AA patients treated with anti-human thymocyte globulin (ATG) immunosuppressive therapy, the response rate at 12 months in the groups with PNH clones was significantly higher than in those without [23].

Although with lower frequency (4.8% PNH clones out of 21 patients), PNH clones have also been identified in patients with chronic myeloproliferative syndromes [15, 24]. It should be investigated whether in these diseases there may be signs and symptoms that may give indications for PNH clone investigation. For example, the presence of Coombs-negative hemolytic anemia, not justified by other causes, could be an indication for clone search. The presence of a PNH clone has also been rarely found in patients with autoimmune hemolytic anemia (AIHA) and, therefore, Coombs-positive [25, 26]. Patients with AIHA might be screened as well if they present with signs and symptoms attributable to the presence of a PNH clone (i.e., unexplained LDH elevation disproportionate to the degree of anemia, atypical thromboses etc.).

The Box 1 summarizes which patients should be tested for PHN clone.

3.2 | Q2. Which Test to Use, and How Do We Define Positivity?

The aims of this question are to establish a standardized method for the qualitative and quantitative detection of the PNH clone, to determine the appropriate assay sensitivity, and to define optimal reporting of clone type and size across different cell lineages.

Different immunophenotypic panels have been developed using monoclonal antibodies (mAbs) with various specificities and conjugated with different fluorochromes, achieving sensitivities

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BOX 1 | Which patients need to be tested?

- In patients presenting with two or more signs or symptoms suggestive of PNH (Table S1), screening for a PNH clone should be considered. This is particularly important when these symptoms are combined, as it increases the likelihood of identifying a clinically significant PNH clone (Table S3).
- In patients with hematologic conditions known to be associated with PNH clones, including DAT-negative hemolysis, idiopathic venous thrombosis (particularly at atypical sites), and primary bone marrow dysfunction (such as aplastic anemia or myelodysplastic syndrome), clinicians should perform testing to rule out the presence of a PNH clone (Table S2).
- For patients diagnosed with AA, even if a PNH clone is not detected at initial diagnosis, regular monitoring for the emergence or growth of a PNH clone is recommended during follow-up.
- In patients with AA treated with immunosuppressive therapy (IST), it is recommended to check for the presence of a PNH clone before treatment and during follow-up. A PNH clone present before ATG treatment is associated with a higher response rate to the therapy.
- In patients below 45 years of age, with any of the above-mentioned clinical features, a PNH clone should be considered with a higher degree of suspicion.

ranging from 0.01% (high-sensitivity tests) to 1% (low-sensitivity tests) of relevant events [27, 28]. For diagnosing classic (hemolytic) PNH in routine clinical practice, a sensitivity of 0.1% is generally acceptable. However, identifying small populations of PNH cells in patients with bone marrow failure syndromes, such as AA and MDS, requires a higher sensitivity up to 0.01% [29].

The most common markers include: CD59 for red blood cells (RBCs) [30], Fluorescent Aerolysin (FLAER), CD24, CD66b and CD157 for neutrophils [30], and FLAER, CD14 and CD157 for monocytes [31].

For allowing an optimal gating of the target population, the most common markers are glycophorin-A (CD235a) and CD45 for red blood cells, CD45, CD33, and CD15 for neutrophils, and CD45, CD33, and CD64 for monocytes [16].

Clone size is assessed by calculating the percentage of GPI-negative cells within the gate of the reference population (neutrophils) [32]. Identification of Type 2 and Type 3 PNH clones can be achieved by analyzing both neutrophils and red blood cells, following current literature recommendations [31, 32].

Analysis of white blood cells (WBCs) by Multiparametric Flow Cytometry (MFC) has advantages in detecting PNH populations and evaluating clone size. Most studies indicate that CD15 and CD64 are more effective than CD33 in selecting neutrophils and monocytes, respectively, and current guidelines recommend using anti-CD15 for neutrophils and CD64 for monocytes [32]. One of the most effective WBC panels includes CD45, CD15, CD64, FLAER, and CD157, enabling detection of PNH populations in neutrophils and monocytes with sensitivities of 0.01% and 0.04%, respectively, using a five-color tube setup [28]. A recent study by Sharma et al. [33] considered using FLAER as a single reagent for detecting PNH clones in WBCs. The validity of an FLAER-only test was compared with a routine panel, which included gating markers (CD45 for the whole leukocyte population, CD15 for granulocytes, and CD64 for monocytes) as well as a combination of markers with FLAER and GPI-linked molecules, such as CD24 and CD14. Testing in 209 patients revealed a PNH clone in 20.1% ($n = 42/209$), with a concordance rate of 100% between both panels. The size of PNH clones detected using the routine panel ranged from 0.01% to 89.7% for granulocytes and from 0.04% to 96.1% for monocytes. In contrast, using the FLAER panel, clone size for granulocytes ranged from 0.01% to 89.6%, and for monocytes ranged from 0.01% to 96.0%.

Statistical analysis showed a significant correlation between PNH clone sizes in granulocytes and monocytes across both panels. These results indicate that FLAER as a stand-alone marker is both specific and sensitive for identifying PNH clones in granulocytes and monocytes, making it effective for high-sensitivity PNH testing. The proposed "FLAER-only" panel was also found to be cost-effective and efficient for PNH testing in two cell lines, which is particularly beneficial in resource-limited clinical settings.

High-sensitivity testing is especially useful for detecting small PNH clones, which are more common in patients with bone marrow failure (BMF) syndromes. The size of the clones may change over time and appears to be positively correlated with response to immunosuppressive treatment [34].

To allow a precise determination of the clone, assuming 20 events or 30 events as the minimum size of a relevant cell event cluster for detection, and 50 events as the minimum for quantitation, the calculation of Limit of Detection (LOD)% is: 20 (or 30)/total number of single population events $\times 100$; and the calculation of Limit of Quantification (LOQ)% is: 50/total number of single population events $\times 100$. Therefore, the minimum number of cells to quantify a given population (e.g., red blood cells, neutrophils, monocytes) depends on the desired sensitivity level: 5000 events for low sensitivity (1.0%), 50 000 events for intermediate sensitivity (0.1%), and 500 000 events for high-sensitivity testing (0.01%).

Current evidence indicates that the actual clone size is best determined on the neutrophil population, which serves as the reference population, rather than by red blood cells. Recent literature suggests incorporating CD71 into the multiparameter panel to evaluate PNH clones in red blood cells, as distinguishing between immature and mature red blood cells may enhance both the qualitative and quantitative assessment of the erythroid lineage [35]. Therefore, the panel used for assessing the PNH clone in the erythroid series should be further optimized.

Regarding the cytometric panel for WBCs, it should be determined whether to use FLAER in combination with multiple

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BOX 2 | Which test to use, and how do we define positivity?

- A high-sensitivity flow cytometry test in neutrophils and erythrocytes should be preferred (lower limit of quantification 0.01%, that is 500000 counted events per each population, to detect 50 GPI-deficient cells). However, in case of high clinical suspicion or detection of a large PNH clone, the number of counted events can be inferior.
- In monocytes or hypocellular samples, the feasible sensitivity may be lower, and the detection threshold can be reliably set at 0.1%–0.5%.
  - When the target population is scarce (e.g., severe neutropenia, low monocyte counts, hypocellular/aplastic bone marrow) and only a lower sensitivity is allowed, a minimum LOD (limit of detection) of 0.1%–0.5% should be pursued.
- LOD and LOQ (limit of quantification) of the test must be reported for each cell population.
- Minimum gating markers should include CD45 (leucocytes) CD15 (neutrophils) CD64 (monocytes) CD235a (erythrocytes).
- The minimum flow panel should include FLAER (neutrophils) and CD59 (erythrocytes).

Optimal markers are the references recommended by the European Society for Clinical Cell Analysis [34].

GPI-linked molecules and gating markers or to use FLAER alone, as suggested in Sharma's 2023 study. This study demonstrated that FLAER is highly specific for GPI-linked molecules and achieves high sensitivity in their detection. However, using FLAER alone may produce data that can be challenging to interpret, especially in the presence of PNH2 and PNH3 clones in neutrophils and monocytes.

Another aspect that requires further definition is the sensitivity threshold for detecting PNH clones. While very small clones do not necessitate treatment, they do require monitoring over time to track their progression.

The Box 2 describes the tests to use to identify PNH clones.

3.3 | Q3. Which Workup Is Requested for Patients With a Newly Detected PNH Clone?

The work-up of a patient carrying a newly diagnosed PNH clone includes the assessment of PNH clone size, PNH clone characteristics, and disease activity. The objectives are to confirm the diagnosis when initial testing was performed at another hospital (by accurately quantifying the PNH clone in RBCs, PMNs, and monocytes) and to assess the clinical significance of the clone, as well as to identify risk factors for major complications such as thrombosis and organ failure. Disease activity is defined by baseline hemolysis and PNH-related signs and symptoms, including thrombosis, and is termed "high" (HDA) if LDH levels are $>1.5 \times$ ULN in the presence of at least one clinical sign/symptom. Further aim is to investigate coexisting conditions, including thromboembolic risk factors or events, aplastic anemia, myelodysplastic syndromes, or idiopathic cytopenias of unknown significance (ICUS) that may require tailored therapeutic strategies.

BOX 3 | Which workup is requested for patients with a newly detected PNH clone.

- Baseline estimation of disease activity requires careful assessment of PNH signs, symptoms, and biomarkers: hemoglobinuria, abdominal pain, dysphagia, erectile dysfunction, fatigue (according to Functional Assessment of Chronic Illness Therapy-FACIT, European Organization for Research and Treatment of Cancer—EORTC, PNH/HMPRO score) [36], LDH, and other hemolysis biomarkers.
- Baseline estimation of thromboembolic risk requires collecting patient history of thromboembolic complications and clinical thromboembolic risk factors (i.e., cardiovascular risk factors, neoplasms, bedrest).
- The workup can be completed by the evaluation of Doppler ultrasound, computed tomography, or magnetic resonance imaging scan to detect venous or arterial thrombosis, and thrombophilia screening in selected cases, and by the assessment of organ damage (i.e., renal failure, pulmonary hypertension).
- Bone Marrow Examination is recommended if an associated BM failure (i.e., AA, MSD) or other hematologic conditions are suspected.

The Box 3 describes the workup required to detect a new PNH clone.

3.4 | Q4. Which Monitoring Is Used in Untreated Patients?

Timely detection of clone expansion and activity (hemolysis, bone marrow failure, and vascular complications) above treatment threshold can drive prompt therapy start to prevent complications and undesirable events that may negatively impact survival and quality of life.

In patients with small clones (usually granulocyte populations $<10\%$) with no evidence of hemolysis or PNH-related disease manifestations, anti-complement therapy may not be required, and it may be appropriate to closely monitor with blood work and clinical assessments. It is recommended that patients with a PNH clone size of more than 0.01% should be monitored depending on the clinical manifestations [20, 25, 37–41]. For instance, in the absence of clinical manifestations, a patient with a clone size $>0.01\%$ but $<1\%$ should be tested every year. In contrast, for patients with a clone size of more than 1% testing should be performed at least once every 6 months [20, 25, 37–41]. However, in case of changes in clinical symptoms, the test should be carried out.

In untreated PNH patients the following conditions may occur and require monitoring: [20, 25, 37–41].

- Hemolysis: Anemia in PNH is often multifactorial and may result from a combination of RBC destruction and lack of production due to underlying bone marrow failure, iron deficiency, or other bone marrow insults.

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- Thrombosis: PNH is characterized by thrombosis in atypical locations. Venous thrombosis (intra-abdominal vessels, e.g., hepatic vein [Budd-Chiari syndrome]) is significantly more common than arterial thrombosis (e.g., cerebral, coronary).
- Renal Failure: intravascular hemolysis in PNH can lead to severe acute hemolytic episodes, which can cause acute renal failure from direct toxicity of free heme in the kidney, or chronic hemolysis may lead to renal iron deposition (renal hemosiderosis), which can interfere with proximal tubule function and cause interstitial scarring and cortical infarcts.
- Pulmonary hypertension may result from nitric oxide depletion in the pulmonary circulation and/or from pulmonary emboli; nevertheless, clinically significant pulmonary hypertension is rare in PNH, and when it does occur, it is often associated with pulmonary emboli.

Bone marrow assessments are generally guided by clinical features and trends of cytopenias for classic hemolytic PNH, whilst in the context of AA or MDS the same monitoring recommended for the underlying disease is required to surveille progression/evolution. Furthermore, in case of PNH associated with MDS, NGS evaluation (i.e., myeloid gene panel) is required for proper classification and risk stratification [42]; on the other hand, in those with classical PNH/associated AA, the utility of NGS myeloid panel is still the object of ongoing research. In fact, while somatic mutations have been described in most aplastic anemia patients, their clinical significance and prognostic value has not been yet clarified [43, 44].

Box 4 Highlights how to monitor untreated patients.

BOX 4 | Which monitoring is used in untreated patients?

In patients with untreated PNH, consider:

- complete blood counts at least including reticulocyte count every 6 months.
- LDH, haptoglobin, total and fractionated bilirubin every 6 months.
- Vitamins and iron assessments every 6 months.
- Clinical and laboratory markers (pro-brain natriuretic peptide–pro-BNP, proteinuria, creatinine) of organ damage, based on patient signs/symptoms and PNH disease activity every 6 months if neutrophil PNH clone size is $>1\%$ or once a year if $>0.01\%$ but $<1\%$.
- Bone marrow function evaluation with bone marrow aspiration (morphology and cytogenetics) and bone marrow biopsy if peripheral blood counts are abnormal at follow-up and in patients with known BMF.
- HLA typing in young patients with associated MDS/BMF.
- Thromboprophylaxis based on PNH disease activity or other conditions that may increase thrombotic risk (i.e., surgery, trauma, infections with hemolytic exacerbations, etc.).
- Vaccinations against capsulated bacteria (*N. meningitidis*, *S. pneumoniae*, *H. influenzae* B) in candidates for anti-complement therapy [45].

3.5 | Q5. Which Monitoring in C-Terminal-Inh and C-Proximal-Inh?

The goals of monitoring in C-terminal and C-proximal inhibition are to assess therapeutic response, to personalize treatment through dose or schedule adjustments or, when necessary, a shift in therapy, and to ensure the timely detection of flare events such as breakthrough hemolysis. Ongoing objectives also include surveillance of the PNH clone trajectory—whether expanding or regressing—together with prompt identification and management of treatment-related adverse effects, PNH-associated complications such as thrombosis, and infectious events requiring specific preventive or therapeutic strategies, including vaccination planning.

The most frequently reported indications for treatment with anti-C5 inhibitor drugs in patients with a documented PNH diagnosis [41, 46, 47] include high disease activity and/or PNH-related complications (e.g., PNH-related thrombosis). The presence of these indications is sufficient to consider treatment with anti-C5 inhibitors regardless of the need for transfusions, as well as the PNH clone size [48]. Monitoring is generally based on periodical clinical assessment of blood counts and hemolytic markers (LDH, unconjugated bilirubin, haptoglobin, and reticulocytes) to evaluate the efficacy of treatment (intravascular hemolysis control, hemoglobin improvement, as per [49]), the need for transfusion, iron/vitamins supplementation, and progression of the associated bone marrow failure (blood counts and reticulocytes).

Flow cytometry PNH clone monitoring is useful in patients receiving anti-C5 inhibitor therapy. In fact, an increase and stabilization of the PNH red cell (RBC) clone is consistent with disease control in treated patients [30]. The percentage of PNH neutrophils/monocytes is usually much higher than the size of PNH RBC [8]. This difference corresponds to the degree of hemolysis and usually correlates with the LDH values. Increased reticulocytes are another marker of hemolysis [50]. Since the immature PNH RBCs are not hemolyzed by activated complement and are not significantly diluted by transfusions, the use of CD71 (transferrin receptor) as a marker of immature RBCs is useful for assessing the real clone size of PNHII and PNH III RBCs [35, 51].

Of note, extravascular hemolysis (EVH) can occur in individuals treated with anti-C5 inhibitor drugs because C3 fragments can opsonize the PNH RBCs, which are not destroyed by the membrane attack complex intravascularly and induce reticuloendothelial destruction in the liver and spleen. Usually, this condition is characterized by persistent anemia, an increase of unconjugated bilirubin and reticulocytes, and a positive DAT for C3d [37]. Flow cytometry analysis of C3 on RBCs provides evidence of selective C3 opsonization of PNH red cells. The proportion of C3 PNH RBCs correlates significantly with the hematologic response to anti-C5 inhibitor treatment [52]. Anti-C5 inhibitor treatment increases the risk of life-threatening *N. meningitidis* infections [53]. Mitigation strategies include vaccination against Men ACYW135 and B, and patient education.

Hemolytic exacerbations (namely, breakthrough hemolysis-BTH) may occur in case of insufficient drug concentrations to

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prevent free C5 activation (pharmacokinetic BTH) or due to complement amplifying conditions such as infections, traumas, and surgery (pharmacodynamic BTH). In both cases, variable degrees of hemoglobin drop and LDH increase may be observed, and BTH may be mild (with hemoglobinuria as the only sign of hemolysis) to severe, requiring transfusion support and modification of C5 inhibitor posology.

A suboptimal response to terminal complement inhibitors may be counteracted by treatment with proximal complement inhibitors targeting C3 or alternative pathway factor B and D [54]. Proximal inhibitors improve the hematological response by achieving broad hemolysis control through the inhibition of C3 [55, 56]. The most appropriate biomarker(s) for monitoring proximal inhibitor–treated patients still rely on clinical signs and symptoms of PNH, hemoglobin levels, and hemolytic markers [57]. Regarding the role of flow cytometry, it allows for the detection and quantification of the amount of C3 fragment deposition on PNH red blood cells [58, 59]. Generally, a decrease in the deposition of C3 fragments on the PNH RBC clones was observed in proximal inhibitor–treated patients [60]. Due to the block of intravascular and extravascular hemolysis in patients treated with proximal complement inhibitors, it is expected that the PNH RBC clone size, detected by flow cytometry, would increase more than when a terminal complement inhibitor is used [61–63].

The increased size of the RBC clone is one of the reasons why breakthrough hemolysis may be more severe in these patients and deserves education and monitoring. A closer interaction among clinicians and flow cytometry laboratory experts is of help to suboptimal responders to anti-C5 therapy and to select patients who may benefit from proximal inhibitors by evaluating the dynamics of C3 deposition on PNH RBCs [64].

Testing of C3 deposition on PNH RBCs by flow cytometry might support estimation of C3-mediated extravascular hemolysis and is currently being investigated.

The Box 5 focuses on monitoring in C-terminal-inh and C-proximal-inh.

BOX 5 | Which monitoring in C-terminal-inh and C-proximal-inh. Statements.

- In PNH patients treated with terminal or proximal complement inhibitors, blood counts and hemolytic markers should be assessed every 8 weeks if LDH is stable $< 1.5/2 \times$ Upper Limit Normal (ULN) and no transfusion is needed. Monitoring should be personalized (at least every 2–4 weeks) if LDH is $> 1.5/2 \times$ ULN and/or symptomatic anemia is reported.
- Prompt testing of blood counts and hemolytic markers is mandatory in PNH patients treated with any complement inhibitors with signs or symptoms of hemolysis to detect BTH.
- In addition to blood counts and hemolytic markers, testing of B12 and folate levels, iron, ferritin and transferrin levels, creatinine, transaminases (ALT/AST), and gamma-glutaryl transferase (GGT) is suggested at least every 4 months.
- Testing of DAT for C3 positivity, and serum C3 and C4 levels is selectively suggested in PNH patients treated with anti-C5 inhibitors developing worsening anemia or increased unconjugated bilirubin and/or increased reticulocyte count.
- Bone marrow evaluation is mandatory in patients developing worsening anemia with reticulocytopenia or additional cytopenia.
- PNH clone analysis every 6 months over the first 2 years and thereafter, once a year for those under anti-C5 inhibitors treatment with stable disease.
- Addition of CD71 in the analysis of the monitored PNH clone is suggested since it significantly improves the ability to detect PNH RBC clone size regardless of treated patients with hemolysis or transfusion-dependent.
- Continuous education regarding the risk of infection and regular check of vaccination status for anti-men ACYW135 and B for anti-C5 as well as for Haemophilus infuenzae B and Pneumococcus for proximal inhibitors should be pursued.
- Treatment adherence should be strictly pursued by physicians caring for patients treated with either terminal or proximal inhibitors. Patients need to be carefully educated to avoid autonomous treatment discontinuation.

4 | Conclusions

Rare complement-related diseases are gaining attention thanks to available targeted therapies, namely anti-complement inhibitors, which dramatically changed the patient's lifespan and quality of life. C5 inhibitors were groundbreaking in their ability to target the terminal complement cascade and reduce hemolysis; as a consequence, diagnostic pathways have been increased, and testing feasibility has expanded [65]. Nevertheless, a large portion of patients receive delayed and incorrect diagnoses [11], and several pending questions arose regarding the optimal patient selection for PNH screening and the optimal monitoring of treated patients. While artificial intelligence-based screenings [66] are being explored and specialist networks [67] synergize the diagnostic efforts in some countries, guidelines and consensus-based recommendations are limited. We, therefore, framed the most relevant clinical questions according to the standard evidence-based methods and provided some practice statements for the most relevant topics in the management of patients with PNH. Consensus rate for the statements was invariably higher than 90%; therefore, we are confident that most of the suggested clinical actions are both scientifically sound and feasible.

Patient selection for screening of rare disorders [68] is a major healthcare problem, and the feasibility of testing may be a hurdle [17]. Even those patients known to face the highest risk of developing PNH, namely those with aplastic anemia, cannot be monitored according to an evidence-based algorithm [69]. It should be noted that PNH belongs to the broader group of hemolytic anemias, that includes congenital (i.e., membrane and enzyme red cell defects) and acquired forms such as autoimmune hemolytic anemias, thrombotic microangiopathies, mechanical hemolytic disorders etc. The most important message

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for the clinical community is to implement the testing for all hemolytic markers in the workup of anemia, since this will be the starting point for diagnosing both common as well as ultrarare forms such as PNH. Thereafter, the panel suggested to test for PNH by using high sensitive flow cytometry all patients with unexplained hemolytic anemia and/or PNH related signs and symptoms, or those subjects suffering from conditions typically associated with PNH clone expansion, such as BM failure. Once the diagnosis of a "PNH clone" is established, it should be classified according to the clinical frame in subclinical, classic hemolytic, or PNH in the context of a BM failure. Baseline evaluation will focus on PNH disease activity, the risk of complications, and the assessment of the potential underlying conditions. Monitoring untreated PNH clones is advised to detect progression to treatment need or disease evolution.

PNH patients receiving complement inhibitors still need careful monitoring of residual or breakthrough hemolysis or disease complications [70]. Monitoring is an important preventive clinical action that is rarely evidence-based. The experts suggested the timing for reassessment of clone size and hemolysis biomarkers to define the response to the current inhibitor as well as the possible occurrence of extravascular hemolysis or breakthrough hemolysis. The latter require special discussion and education with patients and caregivers, as does the need for treatment adherence to both terminal and proximal inhibitors, and the importance of vaccination strategies and prompt referral to medical attention in case of infections. Systematic PNH clone monitoring, as well as bone marrow assessment in case of worsening cytopenia, was also discussed to detect possible disease permutations (evolution into myeloid neoplasms versus PNH clone disappearance).

In conclusion, this manuscript provides useful and feasible support to the clinicians managing PNH at spoke centers and allows residents to build up a strategy for these unique tasks. The GRADE framework allows for embedding indirect evidence into patient-intervention-outcome questions and makes it possible to provide evidence-based recommendations when enough direct data are available.

Author Contributions

B.F., F.B., and M.M. designed the project; all authors contributed to the acquisition, analysis, and interpretation of literature data for the work. All authors contributed to writing the first draft and reviewed the work critically for important intellectual content, approved the final version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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The authors have nothing to report.

Conflicts of Interest

B.F. works as consultant/advisor for Agios, Roche, Sobi, Samsung, Alexion, and Novartis and as speaker from Alexion and Novartis; M.M. provides consultancy to Novartis, Menarini, BMS, Beigene, Roche and works as Speakers Bureau for Otsuka and MSD; S.S. works as advisor and speaker for Alexion, Sobi, Novartis, and Roche; F.B. works as consultant/advisor for Jazz Pharmaceuticals, Laboratoires Delbert, Novartis and as speaker for Astellas, Bristol Myers Squibb, Janssen-Cilag, Servier, Menarini Stemline. Other authors declare no conflicts of interest.

Data Availability Statement

All the results have been reported here, and no other data have been generated in this work.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Prevalence of PNH clones in the listed clinical scenarios. **Table S2:** Prevalence of PNH clone in specific diseases. **Table S3:** Synergic effect of signs and symptoms on the prevalence of PNH clones.

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