Diagnosis of Paroxysmal Nocturnal Hemoglobinuria: Recent Advances

Prabhu Manivannan¹ · Ankur Ahuja² · Hara Prasad Pati³

Received: 13 March 2017 / Accepted: 21 August 2017 / Published online: 8 September 2017
© Indian Society of Haematology & Transfusion Medicine 2017

Abstract Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematopoietic stem cell disorder with its protean clinical manifestations. This is due to partial or complete absence of ‘glycophosphatidyl-inositol-anchor proteins’ (GPI-AP). The main aim of this review is to highlight various diagnostic modalities available, basic principle of each test and recent advances in the diagnosis of PNH. Recently among various tests available, the flow cytometry has become ‘the gold standard’ for PNH testing. In order to overcome the difficulties encountered by the testing and research laboratories throughout the world, International Clinical Cytometry Society has come up with guidelines regarding the indications for testing, protocol for sample collection, processing, panel of antibodies as well as gating strategies to be used, how to interpret the test and reporting format to be used. It is essential to test at least two GPI-linked markers on at least two different lineages particularly on red cells and granulocytes/monocytes. The fluorescent aerolysin combined with other monoclonal antibodies in multicolour flow cytometry offered an improved assay not only for diagnosis but also for monitoring of PNH clones. It is equally important to diagnose this rare entity with high index of suspicion.

Keywords Paroxysmal nocturnal hemoglobinuria (PNH) · Glycophosphatidyl-inositol-anchor proteins (GPI-AP) ·

Flow cytometry · Fluorescent aerolysin (FLAER) · International Clinical Cytometry Society (ICCS)

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, acquired clonal hematopoietic stem cell disease that manifests as hemolytic anemia, bone marrow failure, smooth muscle dystonia and thrombosis [1–5]. PNH originates from a multipotent hematopoietic stem cell that acquires a mutation in a gene called phosphatidylinositol glycan anchor biosynthesis, class A (*PIG-A*) located on chromosome Xp22.1. This leads to partial or absolute deficiency of all ‘glycophosphatidyl-inositol-anchor proteins’ (GPI-AP) [6, 7]. These proteins serve different functions like enzymes, receptors; complement regulators and adhesion molecules [8]. The protein deficiencies such as CD55 (decay accelerating factor, DAF) and CD59 (membrane inhibitor of reactive lysis, MRL) leads to complement mediated cell lysis and other clinical manifestations [1].

Various tests like modified Ham’s test, sucrose lysis test, gel card technique (GCT) and flow cytometry-based testing are available for making diagnosis [9–12]. Recently, in multicolour flow cytometry, bacterial aerolysin tagged with fluorescent antibody (FLAER) was used which binds specifically to the GPI moiety of cell surface GPI-linked molecules and causes lysis of normal but not GPI-deficient PNH cells [13–16]. This review aims at giving brief overview of clinical manifestations of PNH and its pathogenesis, different diagnostic tests available for detection of PNH clone, and its recent advances in recommendations for making its diagnosis.

✉ Hara Prasad Pati
harapati@yahoo.co.in

¹ Department of Pathology, JIPMER, Puducherry, India

² Department of Laboratory Services, Army Hospital for Research and Referral, New Delhi, India

³ Department of Hematology, All India Institute of Medical Sciences (AIIMS), New Delhi, India

Clinical Manifestations of PNH and Its Pathogenesis

The Role of Complement in Intravascular Hemolysis

Most important among the ‘GPI-APs’ are CD55 (DAF) and CD59 (MIRL) which play a vital role in the regulation of complement activation by preventing it from being attached to cell membranes [1–5]. The absence of CD59 from the surface of PNH erythrocytes leads to intravascular hemolysis and hemoglobinuria which manifest as cola-colored urine and subsequently, iron deficiency anemia. There are three pathways by which complement is activated, namely classical, lectin and alternative pathway [5]. Hemolysis in PNH is chronic due to a continuous state of alternate complement pathway activation, but paroxysms may result from increase in complement activation due to infection, surgery, strenuous activity, and excessive alcohol intake [1–5]. Since there are no antibodies involved in this process of hemolysis, PNH should be suspected in all cases of Coombs-negative hemolytic anemia. Usually fatigability is out of proportion to the degree of anemia due to cytokines involved in this process [17]. Depending on the complement sensitivity to lysis, these PNH cells are categorised into type I (normal sensitivity), type II (3–5 times more sensitive) and type III cells (15–25 times more sensitive to lysis) [18].

Nitric Oxide Scavenging in PNH

Nitric oxide plays an important role in maintaining the vascular tone and limiting platelet activation. Intravascular hemolysis in PNH causes free hemoglobin release into plasma which scavenges nitric oxide resulting in various manifestations like fatigue, abdominal pain, dysphagia, male erectile dysfunction and possibly thrombosis [1–5].

Bone Marrow Failure Syndromes

Approximately 30–45% of PNH patients have some kind of bone marrow failure syndromes such as aplastic anemia (AA), myelodysplastic syndrome (MDS); typically refractory anemia and unexplained cytopenia(s) [19]. Mahapatra et al. [20] in their extensive study of 1501 AA patients, PNH clone was detected in 39.7% at diagnosis. Similarly various studies report that at least 40–70% of AA patients have detectable level of PNH clone [21–27].

Two-hit hypothesis model has been proposed to explain the clonal expansion in PNH. The first hit involves the benign event such as PIG-A mutation occurring in hematopoietic stem cells and the second hit involves the immune selection that selectively destroys the normal stem

cells sparing the PNH stem cells. This plays a major role in clonal selection and expansion of the PNH clones [6, 7]. This also explains the possible association of acquired AA with autoimmune basis of the disease and PNH [28–30].

Thrombosis in PNH

Thrombosis is the leading cause of mortality and significant morbidity in PNH patients. It occurs mostly in venous system in 30–40% of patients. The PNH clone size of >50% in granulocytes correlates highly with thrombotic risk. The exact mechanism of thrombosis is not entirely understood, however various postulation have been put forward to explain the association. Nitric oxide depletion, lack of ‘GPI-AP’ such as urokinase receptor and tissue factor pathway inhibitor (TFPI) cause defective fibrinolysis and loss of inhibition of tissue factor [18, 28–30].

Any unexplained thrombosis such as stroke, myocardial infarction, pulmonary emboli and deep vein thrombosis or unusual sites such as hepatic vein thrombosis manifesting as Budd–Chiari syndrome, other intra-abdominal veins such as portal or mesenteric vein thrombosis, cerebral sinus thrombosis, dermal vein thrombosis or thrombosis associated with hemolysis or unexplained cytopenia(s) should be investigated for PNH [31]. Also, a higher failure rate is noted with anticoagulant therapy in a subset of patients with PNH [32].

Classification of PNH

The International PNH interest group (IPIG) established the classification system bringing the clinicians and the researchers together for the diagnosis and quantification of PNH clones. Three subgroups were proposed as follows: (1) Classical PNH is characterised by the clinical evidence of intravascular hemolysis, presence of large granulocyte clone and the majority of the RBCs are of type III (complete ‘GPI-AP’ deficiency) PNH cells; (2) PNH arising in the setting of other bone marrow disorders such as AA and MDS; (3) Subclinical PNH (PNH-sc), highly sensitive flow cytometer assay is required to diagnose this entity because of the presence of smaller clone size along with other bone marrow disorders. Overt symptoms of PNH are not present [3].

Detection of PNH Clones

Various modalities are currently being used to diagnose PNH. In the following sections, we will see about their basic principle, advantages and disadvantages.

Complement Based Test

Modified Ham's test and sucrose lysis test, both are traditional tests, based on the principle that PNH red cells are sensitive to undergo activated complement protein mediated cell lysis. The detection limit of these tests is between 4.2 and 5% [9]. These tests are inexpensive, but require a dedicated technician, laborious and not suitable for routine testing. It is not accurately quantitative, might not give valuable results during hemolytic episodes and following blood transfusion. Moreover, it is not specific, gives false positive results in congenital dyserythropoietic anemia type II, few cases of megaloblastic anemia and autoimmune hemolytic anemia [12, 33].

Gel Card Technique (GCT)

This test is based on antigen–antibody reaction. The advantage is its sensitivity ranges between 2 and 10%, is comparable with complement based test and also simple, inexpensive and easy to interpret [9, 33]. But the major disadvantage is that it is not quantitative, not sensitive to pick up smaller clones, not able to detect PNH cells in a patient who had received blood transfusion in the recent past and during hemolytic episodes [10, 12].

Flow Cytometry Based Test

This utilises monoclonal antibodies tagged with fluorochromes which bind specifically to 'GPI-AP' on peripheral blood cells or FLAER which binds directly to GPI anchor itself [9, 11]. It is rapid, sensitive; gives better assessment of clone size and delineation of type I, II and III cells. This has now become the 'gold standard' test for diagnosis of PNH [12, 33–35]. However, detailed knowledge of various monoclonal antibodies commercially available, their pattern of expression on cells at various stages of maturation, appropriate panel of antibodies to be used and proper gating strategy is required [36, 37].

Pre-analytical Variables

Which is Ideal Sample? Peripheral Blood or Bone Marrow for Analysis

The recent ICCS guidelines recommend the use of peripheral blood for PNH testing. Bone marrow samples should not be used for routine clinical laboratory testing; this is because immature myeloid cells often lack uniform expression of 'GPI-AP' [31]. Also in MDS, often there is altered expression of CD16 on granulocytes and difficult to

interpret test results in the presence of smaller PNH clone [14, 21, 31].

Specimen Collection and Handling

Ideally venous blood should be collected into vacutainer tube containing anticoagulant such as ethylenediamine tetra-acetic acid (EDTA); heparin or acid citrate dextrose (ACD) is also acceptable. The test should be performed within 48 h of sample collection. However if there is delay in testing, samples should be stored at 4 °C for maximum of 1 week. Type III red cells might undergo hemolysis in vitro beyond this period [31].

Analysis of RBC

In PNH, hemolysis is one of the major manifestations of the disease. So analysis of RBC for the expression of various 'GPI-AP' was carried out from the initial experimental studies [34, 35]. It is stressed that while doing sample preparation, particularly for RBC, it is better to avoid excess of protein support during both the incubation and washing step as this causes agglutination of RBC [31]. Similarly in selecting the fluorochromes, phycoerythrin (PE) is recommended when compared to fluorescent isothiocyanate (FITC), as the latter causes agglutination and less distinction between type I, II and III cells [31, 37].

The GPI-linked markers such as CD55 and CD59 are commonly used for analysis of RBC. Simultaneous use of both these markers are advised, because a very rare hereditary disorder in which absence of individual markers CD55 ('Inab' phenotype) and CD59 have been reported [34]. The intensity of CD59 expression is stronger than CD55 and therefore recent guidelines give more weightage for CD59 [31]. Routinely, the gating strategy for RBC is based on light scatter properties such as forward scatter and side scatter (FSC and SSC) in log mode. In order to increase the sensitivity, it is now recommended to use glycophorin A (CD235-FITC). However, care must be taken to ensure adequate washing step to avoid agglutination of RBC when using this marker [36, 37].

Phenotypic mosaicism is the hallmark of the disease as this is manifested as marked variation in the distinction of type I, II and III cells. Therefore, testing of RBCs alone is not recommended because of reduced clone size by hemolysis and recent blood transfusion [31, 38, 39]. In the presence of hemolysis, few authors studied the clone size of reticulocytes stained with CD59 and thiazole orange and found to correlate well with clone size of granulocytes. However, this has no clinical utility and hence not recommended [40, 41]. The advantage with analysis of RBC by flow cytometry is that even after blood transfusion, PNH clone could be picked up however it would underestimate

the clone size, because transfused RBCs show normal expression of CD55 and CD59 [31, 38, 39].

Analysis of WBC

There are three technical protocols available for WBC sample preparation. They are (1) lyse–wash–stain technique, (2) stain–lyse–wash technique and (3) stain–no lyse–no wash technique [36]. In our experience, the first technique which includes initial lysis of RBC by ammonium chloride based reagent, washing with phosphate buffered saline (PBS) followed by staining with monoclonal antibodies gives better result and also recommended by ICCS. This seems to be more economical as it prevents non-specific binding and lesser quantities of monoclonal antibodies used [31].

In cases of AA and MDS, another technical issue to enrich the sample is either by acquiring larger number of events or by using buffy coat because of neutropenia and monocytopenia [31, 37].

The other technical issue that must be considered includes the panel of antibodies and gating strategy used for granulocytes and monocytes. Various initial studies were based on light scatter properties such FSC and SSC for gating of these cell types [35–39]. Our previous experience had demonstrated that in a panel of antibodies (CD55, CD59, CD24 and CD66b), the intensity of CD55 was better than CD59 on granulocytes. The combination of CD24 and CD66b has added advantage of clearly separating granulocytes population from the eosinophils. However significant problem arises in the setting of cytopenia(s) in AA and MDS [21–25]. Therefore, it is now recommended to use non-GPI linked specific lineage markers such as CD15 and CD33/CD64 for granulocytes and monocytes respectively, in addition to CD45 for initial gating strategy. The recent guidelines state that CD64 gives better results when compared to CD33 [31]. In our experience, both these markers are equally good in delineating monocyte population.

Sutherland et al. recommends that two GPI linked antigens are assessed to confirm PNH on two different lineages, most commonly on RBCs and granulocyte/monocyte series. This is because in rare hereditary disorders, absence of specific cell surface antigen causes negativity for that monoclonal antibody tested and in order to increase the sensitivity of the test, double negativity is recommended for two GPI-linked antigens [13]. Therefore in multicolour flowcytometry, in addition to the above markers, one should use CD14 and CD24 for monocytes and granulocytes respectively. The monocytes clone size is generally larger than neutrophils clone size and both correlate well with thrombotic risk [18, 23, 28–30]. Their

clone size is almost always larger than RBC clone size because of hemolysis [31, 38, 39].

In general, lymphocytes are not recommended for PNH testing because of their longer life span and only those lymphocytes originated after the onset of disease will be lacking in ‘GPI-AP’. Therefore, in patients with recent onset of PNH, testing of lymphocytes will give rise to erroneous results [31]. The advantage with the lymphocytes is that it can be used as internal control of PNH testing [36, 37].

Fluorescent Aerolysin (FLAER)

Aerolysin is the toxin secreted by bacterium *Aeromonas hydrophilia*. It is secreted as inactive form, pro-aerolysin, which upon proteolytic cleavage of C-terminal peptide becomes active form. It binds to the cell surface structures, oligomerizes and forms channels resulting in cell lysis [9, 13, 42]. In flow cytometry, mutant form of pro-aerolysin is tagged with fluorescent marker, usually FITC (Alexa Flour 488). This reagent requires activating proteases which is seen in all peripheral blood cells except red cells [14–16].

Using FLAER, various studies could reliably diagnose PNH with high sensitivity and show comparable results with conventional flow cytometric immunophenotyping (FCMI) results, thereby allowing the identification of GPI-negative granulocyte population as small as 0.5% [5, 15, 16, 29]. Since FLAER binds selectively to GPI anchor itself, Brodsky et al. [43] found that FLAER detected similar or higher proportion of PNH monocytes and granulocytes when compared to anti-CD59.

A single tube assay can be performed by combining FLAER with other monoclonal antibodies such as CD45, CD33, and CD14; thereby able to analyse FLAER and the GPI-linked marker CD14 on neutrophils and monocytes simultaneously [31]. In our experience using six color and two laser flow cytometry (BD FACS Canto II), similarly a single tube assay was performed with CD45, CD15, CD33, CD14, CD24 and FLAER (Figs. 1, 2). We compared this single tube assay with two tube assay performed using CD55 and CD59 in one tube and CD16 and CD66b in another tube. Using FLAER based single tube assay, keeping receiver operator curve (ROC) cut-off of 0.7% for neutrophils and 0.9% for monocytes, we could pick up smaller clones of even <1% in four cases of AA and MDS which were otherwise not picked up on two tube assay. Similarly Sreedharanunni et al. [44] emphasised this approach of FLAER based flow cytometric testing to be useful in pediatric AA patients. The authors could demonstrate improved PNH clone detection compared to non-FLAER based screening techniques. Therefore in agreement with other study, FLAER combined with

Fig. 1 Scatter plots of healthy control and respective histograms

multiparameter flow cytometry offered an improved assay not only for diagnosis and monitoring of PNH clones, but also proved to be more economical [15].

It is more robust technique in which the samples can be utilized up to 48 h for testing. Although its use is restricted only to leucocytes, it gives better delineation of type I, II and III cells, particularly if PNH is associated with AA and MDS [21–31]. Nevertheless in the era of complement blockade therapy like eculizumab, the routine analysis of RBC by CD59 cannot be completely replaced by FLAER. This is because after starting therapy with this drug, monitoring of red cells is required which show increase in clone size despite decrease in hemolysis [12, 45].

CD157

CD157 is another GPI-linked structure expressed on both granulocytes and monocytes. In order to provide improved testing algorithm, Sutherland et al. studied whether CD157 could replace the CD24 and CD14 in four-color granulocyte and monocyte assays respectively. This new marker was highly correlated ($R^2 > 0.99$) over a wide range of samples (0.06–99.8% clone size), had similar high level of sensitivity and low background level in the normal samples [46]. The ongoing study in our department with a panel comprised of CD45, CD15, CD64, CD157 and FLAER showed similar results and proved to be more economical (Fig. 3).

Fig. 2 Scatter plots of PNH case showing CD24-FLAER-deficient neutrophils (92.7%) and CD14-FLAER-deficient monocytes (93.6%) were observed. The corresponding *histograms* show predominance of PNH type II cells (81%) and smaller proportion of type III cells

Analysis of Platelets

Up to 10% of the normal platelets, the expression of CD55 and CD59 might be completely absent and in remaining 90%, the expression is generally weak. Therefore, other than research setting, there is no clear recommendation for immunophenotypic analysis of platelets in PNH [37, 38].

Ancillary Investigations

In hemolytic PNH, biomarkers of intravascular hemolysis such as indirect hyperbilirubinemia, decrease in serum haptoglobin, reticulocytosis, and increased serum lactate dehydrogenase (LDH) enzyme are usually additional findings [1–5, 31]. Patients may present with renal failure due to hemoglobinuria and centrifuged urine deposit should be stained with Prussian blue stain to look for hemosiderin pigment in the epithelial cells [1–5].

Fig. 3 Scatter plots of PNH case showing CD157-FLAER-deficient neutrophils (57.4%) and CD157-FLAER-deficient monocytes (74.5%) were observed. The corresponding *histograms* show predominance of PNH type III cells (45%)

Apart from chronic myeloid leukemia, the other condition where leucocyte alkaline phosphatase (LAP) stain would benefit is PNH. Since this is a 'GPI-AP', LAP score is reduced in PNH. But this test is of historical importance only and not widely practised [8]. Finally in patients with unexplained cytopenia(s), bone marrow aspiration and trephine biopsy is required to rule out associated AA and MDS [21–31]. Measurement of D-dimer is very useful in the evaluation and management of thrombotic events associated with PNH [47]. PIGA mutation analysis is confirmatory, however technically difficult and not widely available, mostly done only in research setting [48].

Whom to Test?

The various clinical indications for PNH testing according to the recent ICCS guidelines are highlighted in the Table 1 [31].

When to Test and How Frequently to Test for Follow-Up?

The ICCS guidelines recommend that once a diagnosis of PNH is made, the test should be repeated every 3–6 months initially and then annually thereafter. The basis behind this

Table 1 Clinical indications for PNH testing [31]

Sl. no.	Various clinical indications
1	Intravascular hemolysis as evidenced by hemoglobinuria or elevated plasma hemoglobin
2	Evidence of unexplained hemolysis with accompanying Iron-deficiency/abdominal pain or esophageal spasm/thrombosis/granulocytopenia and/or thrombocytopenia
3	Other acquired Coombs'-negative, non-schistocytic, non-infectious hemolytic anemia
4	Thrombosis with unusual features Unusual sites such as hepatic veins (Budd–Chiari syndrome)/other intra-abdominal veins (portal, splenic, splanchnic)/cerebral sinuses/dermal veins/with signs of accompanying hemolytic anemia/with unexplained cytopenia
5	Evidence of bone marrow failure Suspected or proven aplastic or hypoplastic anemia, refractory cytopenia with unilineage dysplasia, other cytopenias of unknown etiology after adequate workup

frequent monitoring is that since PNH is an acquired disease, the clone size varies over a period of time [31]. In approximately 20–50% of the cases over a 5-year period, the clone remains stable, whereas in other 10–15% of cases, the clone size either increases or decreases with time or eventually disappears altogether [49, 50]. This monitoring is essential because patients with very small clone size are followed with observation alone. On the contrary, in the era of eculizumab therapy, treatment should be started at the earliest possible moment to prevent the complications, if the clone size increases on follow-up [1–5, 45, 51].

How to Test?

The ICCS guidelines have described routine analysis in clinical laboratories as detecting abnormal PNH clone of $\geq 1\%$ and high sensitivity analysis, in which as few as 0.01% clones are detected. The number of events to be analysed for routine and higher sensitivity analysis are 5000–10,000 and at least 1,00,000 specific cell events respectively. The sensitivities in such analysis should be 1 and 0.01% respectively. High-sensitivity analysis is the preferred method for picking up PNH-sc [14, 17, 31]. The detection of any clone size is important in these scenarios, because this serves as a biomarker for high likelihood of response to immunosuppressive therapy [25].

How Results Should be Reported?

Most of the hematopathologists are well aware of this entity. However there is lack of consensus in the testing strategy and reporting terminology. This was highlighted in

the study conducted by United Kingdom National External Quality Assurance Schemes (UK NEQAS) [37].

It is advisable not to use the terminology such as 'positive or negative for PNH'. It is good laboratory practice to state whether PNH clone is present or absent in the report. For red cells, it is recommended to provide type I, II and III clone size [31]. With respect to granulocytes, the clinical significance of type II cells is uncertain. However, type III cells $>50\%$ are associated with significant risk for thrombosis [18, 28–31]. It is also recommended that at least two different cell lineages should be tested for confirmatory diagnosis [31]. When the laboratory is handling the follow-up samples, the report should include the previous clone size and should highlight any changes in clone size. Ideally good communication with treating oncologist/hematologist is required for better management strategy [32].

Quality Control and Validation

It is always encouraged to analyse large number of samples from normal subjects before establishing the test in the laboratory. It is also recommended for the laboratory to establish their sensitivity for red cells and granulocytes/monocytes [31, 37]. Since this disease is a rare entity and commercial controls are costly, to be more economical, the practical way is to use other cells such as lymphocytes as internal control because of their longer life span [36].

The laboratory should take part in external quality assurance program. Currently, there are two schemes available: (1) The College of American Pathologists scheme and (2) The United Kingdom National External Quality Assurance Scheme (UK NEQAS). The former assesses only red cell assay, whereas latter scheme is for leukocyte immunophenotyping [37]. Most of the laboratories faced significant problems in terms of both false positive and false negative test results which could have

resulted in serious clinical consequences. In order to overcome these issues, recently ICCS has come up with guidelines for every aspect of testing strategy and uniform reporting format to be used throughout the world [31]. Since this condition is rare, it is recommended to exchange the samples between the laboratories [37].

Conclusion

PNH is a life threatening disorder with various clinical manifestations. In this review, we have highlighted various techniques available with their basic principles, recent advances in diagnosis, advantages and their limitations. Currently the 'gold standard' test for making early diagnosis is flow cytometry with FLAER based testing [13–16]. In order to overcome difficulties encountered in PNH testing as well as to have uniform reporting format throughout the world, ICCS has come up with recent guidelines that should be followed in our day to day practice [31]. Given the rarity of this disease with its protean manifestations, high index of suspicion is required to make early diagnosis to prevent complications and appropriate management [1–5, 45, 51].

Acknowledgements Currently Dr. Prabhu Manivannan is working as Assistant Professor in the department of Pathology, JIPMER, Puducherry would like to extend his acknowledgement to all laboratory technicians and Dr. Roopam Deka (Senior Resident), All India Institute of Medical Sciences (AIIMS), New Delhi for their dedicated work and for contributing CD157 plot for this manuscript. He would also like to acknowledge his wife Mrs. Manjari for final proof-reading of this manuscript. Dr. (Lt. Col) Ankur Ahuja is currently working as Assistant Professor in the department of Laboratory services, Army hospital Research and Referral, New Delhi. He would like to acknowledge his department as well as the hospital for letting him to contribute to this manuscript.

Compliance with Ethical Standards

Conflict of interest None.

References

- Parker C, Omine M, Richards S et al (2005) Diagnosis and management of paroxysmal nocturnal hemoglobinuria. *Blood* 106:3699–3709
- Pu JJ, Brodsky RA (2011) Paroxysmal nocturnal hemoglobinuria from bench to bedside. *Clin Transl Sci* 4:219–224
- Richards SJ, Hill A, Hillmen P (2007) Recent advances in the diagnosis, monitoring and management of patients with paroxysmal nocturnal haemoglobinuria. *Cytom B Clin Cytom* 72:291–298
- Hill A, Richards S, Hillmen P (2007) Recent developments in the understanding and management of paroxysmal nocturnal hemoglobinuria. *Br J Haematol* 137:181–192
- Brodsky RA (2008) Advances in the diagnosis and therapy of paroxysmal nocturnal hemoglobinuria. *Blood Rev* 22:65–74
- Hu R, Mukhina GL, Piantadosi S et al (2005) PIG-A mutations in normal hematopoiesis. *Blood* 105:3848–3854
- Prince GM, Nguyen M, Lazarus HM et al (1995) Peripheral blood harvest of unaffected CD34+ CD38– hematopoietic precursors in paroxysmal nocturnal hemoglobinuria. *Blood* 86:3381–3386
- Zacks MA, Garg N (2006) Recent developments in the molecular, biochemical and functional characterization of GPI8 and the GPI-anchoring mechanisms. *Mol Membr Biol* 23:209–225
- Bain BJ, Win N (2006) Acquired haemolytic anaemias. In: Lewis SM, Bain BJ and Bates I (eds) *Dacie and Lewis Practical Haematology*. Philadelphia; 10th ed, pp 239–266
- Zupanska B, Bogdanik B, Pyl H (2002) A gel microtyping system for diagnosis of paroxysmal nocturnal hemoglobinuria. *Immunohematology* 18:9–12
- Hall SE, Rosse WF (1996) The use of monoclonal antibodies and flow cytometry in the diagnosis of paroxysmal nocturnal hemoglobinuria. *Blood* 87:5332–5340
- Madkaikar M, Gupta M, Jijina F, Ghosh K (2009) Paroxysmal nocturnal haemoglobinuria: diagnostic tests, advantages, & limitations. *Eur J Hematol* 83:503–511
- Sutherland DR, Kuek N, Davidson J et al (2007) Diagnosing PNH with FLAER and multiparameter flow cytometry. *Cytom B Clin Cytom* 72:167–177
- Sutherland R, Keeney M, Illingworth A (2012) Practical guidelines for the high-sensitivity detection and monitoring of paroxysmal nocturnal hemoglobinuria clones by flow cytometry. *Cytom B Clin Cytom* 82:195–208
- Brodsky RA, Mukhina G, Li S et al (2000) Improved detection and characterization of paroxysmal nocturnal hemoglobinuria using fluorescent aerolysin. *Am J Clin Pathol* 114:459–466
- Peghini PE, Fehr J (2005) Clinical evaluation of an aerolysin-based screening test for paroxysmal nocturnal hemoglobinuria. *Cytom B Clin Cytom* 67:13–18
- Movalia MK, Weitz IC, Lim SH, Illingworth A (2011) Incidence of PNH clones by diagnostic code utilizing high sensitivity flow cytometry. *Blood (Abstracts from the 2011 American Society of Hematology Annual Meeting)* 118:Abstract 1033
- Latour RP, Mary JY, Salanoubat C et al (2008) Paroxysmal nocturnal hemoglobinuria: natural history of disease subcategories. *Blood* 112:3099–3106
- Schrezenmeier H, Muus P, Socie G et al (2014) Baseline characteristics and disease burden in patients in the International Paroxysmal Nocturnal Hemoglobinuria Registry. *Haematologica* 99:922–929
- Mahapatra M, Singh PK, Agarwal M et al (2015) Epidemiology, clinico-haematological profile and management of aplastic anaemia: AIIMS experience. *J Assoc Physicians India* 63:30–35
- Wang SA, Pozdnyakova O, Jorgensen JL et al (2009) Detection of paroxysmal nocturnal hemoglobinuria clones in patients with myelodysplastic syndromes and related bone marrow diseases, with emphasis on diagnostic pitfalls and caveats. *Haematologica* 94:29–37
- Mukhina GL, Buckley JT, Barber JP et al (2001) Multilineage glycosylphosphatidylinositol anchor deficient hematopoiesis in untreated aplastic anaemia. *Br J Haematol* 115:476–482
- Sachdeva MU, Varma N, Chandra D et al (2015) Multiparameter FLAER-based flow cytometry for screening of paroxysmal nocturnal hemoglobinuria enhances detection rates in patients with aplastic anemia. *Ann Hematol* 94:721–728
- Wang H, Chuhjo T, Yamazaki H et al (2001) Relative increase of granulocytes with a paroxysmal nocturnal hemoglobinuria phenotype in aplastic anemia patients: the high prevalence at diagnosis. *Eur J Haematol* 66:200–205
- Wang H, Chuhjo T, Yasue S et al (2002) Clinical significance of a minor population of paroxysmal nocturnal hemoglobinuria-type cells in bone marrow failure syndrome. *Blood* 100:3897–3902

26. Agarwal R, Chapple P, Brown M et al (2015) Analysis of abnormal clones by the fluorescent aerolysin method in paroxysmal nocturnal haemoglobinuria and other marrow disorders. *Int J Lab Hematol* 37:14–21
27. Maciejewski JP, Rivera C, Kook H et al (2001) Relationship between bone marrow failure syndromes and the presence of glycosylphosphatidylinositol anchored protein deficient clones. *Br J Haematol* 115:1015–1022
28. Young NS, Abkowitz JL, Luzzatto L (2000) New insights into the pathophysiology of acquired cytopenias. *Hematology Am Soc Hematol Educ Program*, pp 18–38
29. Brodsky RA (2006) New insights into paroxysmal nocturnal hemoglobinuria. *Hematology Am Soc Hematol Educ Program*, pp 24–28
30. Bessler M, Hiken J (2008) The pathophysiology of disease in patients with paroxysmal nocturnal hemoglobinuria. *Hematology Am Soc Hematol Educ Program*, pp 104–108
31. Borowitz MJ, Craig FE, DiGiuseppe JA, On behalf of the Clinical Cytometry Society et al (2010) Guidelines for the diagnosis and monitoring of paroxysmal nocturnal hemoglobinuria and related disorders by flow cytometry. *Cytom B Clin Cytom* 78:211–230
32. Sharma VR (2013) Paroxysmal nocturnal hemoglobinuria: pathogenesis, testing and diagnosis. *Clin Adv Hematol and Oncol*. 11:2–8
33. Gupta R, Pandey P, Choudhry R et al (2007) A prospective comparison of four techniques for diagnosis of paroxysmal nocturnal hemoglobinuria. *Int J Lab Hematol* 29:119–126
34. Oelschlaegel U, Besson I, Arnoulet C et al (2001) A standardized flow cytometric method for screening paroxysmal nocturnal haemoglobinuria (PNH) measuring CD55 and CD59 expression on erythrocytes and granulocytes. *Clin Lab Haematol* 23:81–90
35. Piedras J, López-Karpovitch X (2000) Flow cytometric analysis of glycosylphosphatidylinositol-anchored proteins to assess paroxysmal nocturnal hemoglobinuria clone size. *Cytometry* 42:234–238
36. Hernandez-Campo PM, Almeida J, Sanchez ML et al (2006) Normal patterns of expression of glycosylphosphatidylinositol-anchored proteins on different subsets of peripheral blood cells: a frame of reference for the diagnosis of paroxysmal nocturnal hemoglobinuria. *Cytom B Clin Cytom* 70:71–81
37. Richards SJ, Whitby L, Cullen MJ et al (2009) Development and evaluation of a stabilized whole-blood preparation as a process control material for screening of paroxysmal nocturnal hemoglobinuria by flowcytometry. *Cytom B Clin Cytom* 76:47–55
38. Richards SJ, Rawston AC, Hillmen P (2000) Application of flow cytometry to the diagnosis of paroxysmal nocturnal hemoglobinuria. *Cytometry* 42:223–233
39. Richards SJ, Hillmen P (2002) Immunophenotypic analysis of PNH cells. *Curr Protoc Cytom*. doi:10.1002/0471142956.cy0611s20
40. Navenot JM, Muller JY, Blanchard D (1998) Investigation of the survival of paroxysmal nocturnal hemoglobinuria red cells through the immunophenotyping of reticulocytes. *Transfusion* 38:337–342
41. Ware RE, Rosse WF, Hall SE (1995) Immunophenotypic analysis of reticulocytes in paroxysmal nocturnal hemoglobinuria. *Blood* 86:1586–1589
42. Diep DB, Nelson KL, Raja SM et al (1998) Glycosylphosphatidylinositol anchors of membrane glycoproteins are binding determinants for the channel-forming toxin aerolysin. *J Biol Chem* 273:2355–2360
43. Brodsky RA, Mukhina GL, Nelson KL et al (1999) Resistance of paroxysmal nocturnal hemoglobinuria cells to the glycosylphosphatidylinositol-binding toxin aerolysin. *Blood* 93:1749–1756
44. Sreedharanunni S, Sachdeva MU, Bose P et al (2016) Frequency of Paroxysmal nocturnal hemoglobinuria clones by multiparametric flow cytometry in pediatric aplastic anemia patients of Indian Ethnic origin. *Pediatr Blood Cancer* 63:93–97
45. Parker CJ (2016) Update on the diagnosis and management of paroxysmal nocturnal hemoglobinuria. *Hematology Am Soc Hematol Educ Program*, pp 208–216
46. Sutherland DR, Acton E, Keeney M et al (2014) Use of CD157 in FLAER-based assays for high-sensitivity PNH granulocyte and PNH monocyte detection. *Cytom B Clin Cytom* 86:44–55
47. Weitz IC, Razavi P, Rochanda L et al (2012) Eculizumab therapy results in rapid and sustained decreases in markers of thrombin generation and inflammation in patients with PNH independent of its effects on hemolysis and microparticle formation. *Thromb Res* 130:361–368
48. Wanachiwanawin W, Siripanyaphinyo U et al (2006) A Cohort study of the nature of paroxysmal nocturnal hemoglobinuria clones and PIG-A mutations in patients with aplastic anemia. *Eur J Haematol* 76:502–509
49. Sugimori C, Mochizuki K, Qi Z et al (2009) Origin and fate of blood cells deficient in glycosylphosphatidylinositol-anchored protein among patients with bone marrow failure. *Br J Haematol* 147:102–112
50. Raza A, Ravandi F, Rastogi A et al (2014) A prospective multicentre study of paroxysmal nocturnal hemoglobinuria cells in patients with bone marrow failure. *Cytom B Clin Cyto* 86:175–182
51. Mayo VM, Mukhina GL, Garrett ES et al (2004) Natural history of paroxysmal nocturnal hemoglobinuria using modern diagnostic assays. *Br J Haematol* 126:133–138