



Clinico-Hematological Profile of Paroxysmal Nocturnal Hemoglobinuria in Indian Patients: FLAER Flow Cytometry Based Experience from an Indian Tertiary Care Centre

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Abstract PNH is a rare disease with wide spectrum of intra-vascular hemolysis and thrombosis to sub-clinical PNH clones. We aimed to study the clinico-hematological profile and clone size on granulocytes and monocytes of PNH patients classified as per International PNH Interest Group recommendations. A retrospective analysis of clinico-hematological profile of 112 PNH clone positive patients by FLAER based flow cytometry between January and September 2017 done and classified into classical PNH, PNH with aplastic anemia or myelodysplastic syndrome (PNH-AA/MDS) and sub-clinical PNH clones (PNH-sc). Of 112 patients, majority were PNH-sc (62) followed by PNH-AA/MDS (34) and classical PNH (16). The commonest clinical feature was anemia in all 3 groups followed by jaundice (87.5%) in classical PNH and fever in

PNH-AA/MDS (64.7%) and PNH-sc (48.4%). Thrombosis was present in 25% (4/16) classical PNH and 2.9% (1/34) of PNH-AA/MDS. The mean hemoglobin, reticulocyte count and LDH was higher in classical PNH. Bone marrow was predominantly hypercellular in classical PNH (11/16) and hypocellular in PNH-AA/MDS (31/34) and PNH-sc (50/62) with dyserythropoiesis predominantly in PNH-AA/MDS (83.8%) and PNH-sc (74.1%). Marrow iron was reduced in 62.2% classical PNH contrary to increased in PNH-BMF (58%) and PNH-sc (91%). The mean clone size in PNH-sc was significantly lower with > 50% in 16.2% patients. Three patients with MDS-MLD and MDS-MLD-RS in PNH-sc had > 80% clone on granulocytes and monocytes. Most PNH patients in Indian setting are PNH-sc with significantly lower clone, however, a clone size > 50% is not uncommon in Indian PNH-sc.

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Introduction

PNH is a dreadful hematological disease with a wide disease spectrum ranging from classical PNH presenting with intra-vascular hemolysis and thrombosis to bone marrow failure syndromes. With the advent of more sensitive FLAER based flow cytometric techniques, it is now possible to detect subclinical PNH clones in patients with other bone marrow failure syndromes such as aplastic anemia (AA) and myelodysplastic syndrome (MDS) [1]. The International PNH interest group recommends to classify patients into 3 groups—(a) classical PNH, which includes patients with hemolysis and thrombosis. (b) PNH in the setting of other bone marrow failure syndromes

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(c) subclinical PNH, in which patients have small clones without any evidence of hemolysis or thrombosis [2]. It is difficult to classify all cases accurately in these 3 groups because bone marrow failure is the underlying pathology in almost all cases of PNH.

The clinical presentation depends on the size of the PNH clone. Patients with a clone size of 3–5% PNH III RBCs and 20–25% on granulocytes and monocytes usually have some biochemical evidence of hemolysis. However, gross hemoglobinuria is usually seen when clone of PNHIII RBCs is more than 20% corresponding to a 60% clone on granulocytes and monocytes [3]. Thrombosis, the most common cause of mortality in PNH, is usually seen in patients with a large clone size (> 50%) on granulocytes [4].

Our study is a retrospective study of the clinical characteristics and laboratory parameters of patients detected to have PNH positive clone by FLAER based flow cytometry at our centre.

Materials and Methods

We retrieved the files of all patients detected to have PNH clones by FLAER based flow cytometry at our centre from the period of January 2017 to September 2017. These patients were mostly referred to us for evaluation of hemolytic anemia, refractory anemia, suspected aplastic anemia or for investigating the cause of thrombosis. The PNH clone on these patients was detected by flow cytometry on 3 mL peripheral blood collected in EDTA evacuated blood collection tubes and processed within 1 h of sample collection.

Stain-lyse-wash technique was used for assessing PNH clone in leukocytes. However, for cases with ANC < 500/cu mm, bulk lysis was done using 1 mL of blood. Depending on the TLC, blood was taken (total volume made to 100 µL with normal saline) in BD Falcon tubes to which pre-titrated antibody cocktail comprising of CD45 (PerCP-Cy5.5), CD15 (APC), CD64 (PE-Cy7), CD14 (APC-Cy7), CD24 (PE) and FLAER (FITC) was added for 30 min and samples were incubated in dark for 30 min. After this, 2 mL commercial FACS lyse solution was added to the tube and vortexed after standing for 10 min followed by centrifugation at 1300 rpm for 5 min. The supernatant was then discarded and 2 mL sheath fluid (phosphate buffered saline) was added followed by immediate vortexing and centrifugation at 1300 rpm for 5 min. The supernatant was decanted and sample acquired immediately after addition of 0.5 mL phosphate buffered saline. The study was carried out on BD FACS CANTO II two laser 6 coloured flow cytometer. Acquiring was done till 10,000 events in neutrophils window and 5000 events in

monocytes window were acquired. Analysis was done using BD FACS DIVA software version 8. FLAER used in our study was from Cedarlane corporation, Ontario, Canada.

FLAER was used in combination with CD24 to detect FLAER-negative, CD24-negative, GPI-deficient granulocytes gated using a sequential combination of light scatter (to remove debris), CD45, and bright CD15 expression. The modified monocyte assay used a combination of FLAER and CD14 to detect FLAER-negative, CD14-negative GPI-deficient monocytes gated using a sequential combination of light scatter, CD45 and bright CD64 staining.

The files of these patients were retrieved and relevant information on clinical presentation and laboratory parameters was collected. The clinical information included the presenting clinical features with duration of the disease and evidence of organomegaly. The laboratory parameters included complete blood count, peripheral smear with corrected reticulocyte count, urine for hemosiderin, biochemical evidence of hemolysis like serum bilirubin, liver enzymes and LDH, urine for hemosiderin, renal function tests, bone marrow examination and flow cytometry using FLAER for PNH clones on granulocytes and monocytes. Patients were then classified into 3 groups—(1) classical PNH, patients with evidence of hemolysis and thrombosis in the absence of bone marrow failure syndrome like Aplastic anemia or MDS (2) PNH in the setting of Aplastic Anemia or Myelodysplastic Syndrome (PNH-AA/MDS), which included patients with evidence of hemolysis or thrombosis with evidence of Aplastic Anemia or MDS (3) Patients with bone marrow failure syndrome (AA or MDS) detected to have sub-clinical PNH clones (PNH-sc).

Results

A total of 112 patients were found to have PNH positive clone on granulocytes and monocytes during the study period. The mean age of our study population was 30.7 years with 36 females and 71 males. Of 112 patients, 16 were classified as classical PNH, 34 as PNH in the setting of bone marrow failure syndromes (PNH-AA/MDS) and 62 as bone marrow failure syndromes (AA/MDS) with sub-clinical PNH clones. The salient clinical and laboratory features of the 3 groups is summarised in Tables 1 and 2 respectively.

In the classical PNH group, the age of our patients ranged from 16 to 54 years with a mean age of 30 years. 62.5% (10/16) of these patients were males with a male:female ratio of 1.6:1. The most common clinical feature in this group was anemia seen in 100% patients followed by

Table 1 Clinical characteristics of PNH patients

	Classical PNH n (%) n = 16	PNH in AA/MDS n (%) n = 34	AA/MDS with sub-clinical PNH clones n (%) n = 62
Anemia	16 (100)	34 (100)	57 (91.9)
Bleeding	1 (6.3)	9 (26.5)	30 (35.5)
Fever	6 (37.5)	22 (64.7)	22 (48.4)
Jaundice	14 (87.5)	19 (55.8)	–
Cola coloured urine	11 (68.8)	12 (35.3)	–
Pain abdomen/dysphagia	7 (43.8)	13 (38.2)	8 (12.9)
Thrombosis	4 (25)	1 (2.9)	–
Hepatomegaly	5 (31.3)	8 (23.5)	–
Splenomegaly	6 (37.5)	9 (26.5)	–

Table 2 Laboratory parameters of PNH patients

	Classical PNH n = 16	PNH in AA/MDS n = 34	AA/MDS with sub-clinical PNH clones n = 62
Mean Hb (g/dL)	6.3	4.9	5.2
Mean ANC (/cu mm)	2362	670	585
Mean platelet count (/cu mm)	160,000	73,000	30,000
Mean CRC (%)	9.8	5.8	0.8
Serum unconjugated bilirubin (mg/dL)	2.9	1.4	0.4
Serum OT/PT/ALP (U/L)	115/34/213	118/34/207	52/24/201
Mean urea/creatinine (mg/dL)	32/0.8	20/0.7	22/0.6
Mean uric acid (mg/dL)	4.9	3.9	3.2
Mean LDH (U/L)	2192	1732	212
Bone marrow cellularity	Hypercellular-68% Normocellular-32%	Normocellular-9% Hypocellular-91%	Hypocellular-80.6% Normocellular-19.4%
M:E ratio (mean)	1:3.5	1:3	1:1.5
Dyserythropoiesis	62.5%	83.8%	74.1%
Iron stores (Perls stain)	Reduced-62.2% Normal-13% Increased-24.8%	Reduced-12% Normal-30% Increased-58%	Normal-9% Increased-91%
Granulocytes (mean clone size)	89.6%	65.6%	22.4%
Monocytes (mean clone size)	87.9%	57.4%	24.2%

jaundice in 87.5% (14/16) and cola-coloured urine in 68.6% (11/16). One-third of the patients had hepatomegaly or splenomegaly with hepato-splenomegaly in 31.2% (4/16). Thrombosis was present in 25% (4/16) patients involving hepatic vein (2), portal vein (1) and cortical vein (1). The hemoglobin (Hb) in this group ranged from 2.1 to 9.8 g/dL (mean Hb-6.3 g/dL), Absolute Neutrophil Count (ANC) ranged from 1020 to 4482/cu mm (mean-2362/cu mm) and platelet count was in the range of 80,000–198,000/cu mm with a mean platelet count of 163,000/cu mm. The corrected reticulocyte count (CRC)

ranged from 2.8 to 25% with a mean of 9.8%. The unconjugated bilirubin was in the range of 1.1–3.2 mg/dL (mean-2.9 mg/dL) with LDH ranging from 442 to 2935 U/L (mean-2192 U/L). Bone marrow was hypercellular (11/16) or normocellular (5/16) with erythroid hyperplasia in all cases. Out of 16 cases, 12 showed dyserythropoiesis in < 10% erythroid cells and 2 showed significant dyserythropoiesis. Majority of the patients (62.2%) had reduced bone marrow iron stores on Perls stain (grade 0–1). The clone size in granulocytes ranged from 37.7 to 98.8% (mean-89.6%) and from 32.2 to 96.4% (mean-87.9%) in

monocytes. All 4 patients with thrombosis had a clone size on granulocytes and monocytes of > 80%.

In our second group of 34 patients with PNH in the setting of bone marrow failure syndromes, all patients had Aplastic anemia (PNH-AA). Most of the patients (21/34) were males with male: female ratio of 1.6:1 and a mean age of 29.2 years. The most common clinical feature was anemia (100%) patients followed by fever in 64.7% (22/34) and jaundice in 55.8% (19/34). Only 1 patient in this group had a DVT. 26.4% (9/34) of patients had a spleen palpable ranging from just a palpable tip to 1–2 cm below costal margin. Mild hepatomegaly was also seen in 23.5% (8/34) patients. The laboratory parameters of this group ranged as follows—Hb-2.1–7.8/dL (mean-4.9 g/dL), ANC-117–1020/cu mm (mean-670/cu mm), platelets-10,000–180,000/cu mm (mean-73,000/cu mm), CRC-1.5–14% (mean-5.8%), serum unconjugated bilirubin-0.6–1.8 mg/dL (mean-1.4 mg/dL) and LDH-872–2935 U/L (mean-1732 U/L). Of all 34 patients, 91% (31/34) had a hypocellular marrow with erythroid hyperplasia in 91.1% (31/34) and dyserythropoiesis in 83.8% (26/34). Significant dyserythropoiesis (> 10%) was observed in 47% (16/34) patients. Bone marrow iron stores were reduced in only 12% patients. The clone size on granulocytes ranged from 36.7 to 97.6% (mean-65.6%) and was between 36 and 98% (mean-57.4%) on monocytes. A clone size of 92.8% on granulocytes and 90.4% on monocytes was observed in the single patient with thrombosis in this group.

In the third group of 62 patients of Aplastic anemia (AA)/Myelodysplastic syndrome (MDS) with sub-clinical PNH clones, 55 patients were of AA and 7 had MDS. 61.2% (38/62) were males with a male: female ratio of 1.6:1 and a mean age of 29.2 years. The most common clinical feature in this group was anemia in 91.9% (57/62) followed by fever in 48.4% (30/62) and bleeding in 35.5% (22/62) patients. None of the patients had a history of cola coloured urine, jaundice, hepatomegaly, splenomegaly or thrombosis at presentation. The laboratory parameters of this group ranged as follows—Hb-2.9–6.4 g/dL (mean-5.2 g/dL), ANC-10–1420/cu mm (mean-585/cu mm), platelet count-10,000–150,000/cu mm (mean-30,000/cu mm), CRC-0.5–2% (mean-0.8%), serum unconjugated bilirubin-0.3–0.7 mg/dL (mean-0.4 mg/dL), LDH-192–224 U/L (mean-212 U/L). In this group, 80.6% (50/62) patients had age-adjusted marrow cellularity < 25% and 9.6% (9/62) had marrow cellularity in the range of 25–40%. All the 7 patients of MDS had normocellular bone marrow. Mild erythroid prominence (median M:E ratio-1:1.5) was seen in 86.9% (20/62) patients with mild dyserythropoiesis (< 10%) in 74.1% (46/62) cases. Majority of patients (91%) had grade 4–6 iron stores on Perls stain. The clone size on granulocytes ranged from 1 to 92.4% (mean-22.4%) and clone size on monocytes ranged from 1.1 to 88.4%

(mean-24.2%). In the MDS sub-group, 57.1% (4/7) patients had small PNH clones (< 10%) on granulocytes and monocytes. However, two patients had MDS-MLD (significant dyspoiesis in all 3 lineages) with PNH clone of 88.6% on granulocytes and 86.2% on monocytes and 80.6% on granulocytes and 82.4% on monocytes respectively. Another patient with 42% ring sideroblasts (RS) and MDS with single lineage dysplasia (MDS-SLD-RS) also had clone size > 80% (84.1% on granulocytes and 82% on monocytes). None of these patients had any clinical or laboratory evidence of hemolysis or thrombosis.

In our study group, 14 patients of < 18 years were diagnosed as AA with sub-clinical PNH clones. The majority of patients were males (11/14) with the age ranging from 10 to 18 years. None of these patients had any evidence of hemolysis or organomegaly. The clone size of these patients ranged from 1.8 to 82.4% on granulocytes and 1.2 to 86.6% on monocytes.

Discussion

PNH is a rare hematological disease with estimated worldwide incidence of 1–1.5 cases per million individuals [5]. However, studies from Asian subcontinent report higher incidence of this disease than Western countries [6, 7]. The disease tends to affect females (54.4%) more frequently than males in the age group of 30–59 years [6]. We, however, have a higher prevalence of the disease in males (63.3%) with median age at diagnosis being 30 years. This correlates with comparative meta-analysis from Asia which also report a higher prevalence in males (approx 55.9%) [7]. The higher incidence could be explained by the high incidence of aplastic anemia in our country compared to the West which has a higher incidence in males with an earlier mean age of onset of the disease i.e. 25 years [8]. PNH is rare in children [9]. In our study, 12.5% (14/112) patients were < 18 years of age and were diagnosed as AA with sub-clinical PNH clones.

Our patients were classified into 3 groups as per recommendations of the International PNH interest group—classical PNH, PNH in the setting of other bone marrow failure syndromes and subclinical PNH, in which patients have small clones without any evidence of hemolysis or thrombosis [2]. The majority of our patients belonged to the group of AA/MDS with sub-clinical PNH clones (55.3%) followed by PNH-AA (30.3%) and classical PNH (14.4%). Most of our cases of PNH presented with bone marrow failure rather than hemolysis. The reason why bone marrow failure was the dominant presentation in our study can be explained because of higher prevalence of Aplastic anemia in our country and Asia [8, 10, 11].

The most common presenting clinical feature in all the groups was anemia. Fever was the second most clinical presentation in PNH-AA/MDS and AA/MDS with sub-clinical PNH clones. However, patients with classical PNH presented more frequently with jaundice and cola coloured urine. Thrombosis was present in 25% of patients with classical PNH which is similar to the incidence in Western countries (30%). However, a previous comparative analysis between Asian and patients from the West reported a lower frequency of thrombotic events in Asians (< 15%) [7]. Only 1 of 34 (2.9%) patients developed thrombosis in the PNH-AA/MDS group.

Patients with classical PNH had a higher mean Hb, ANC and platelet count than patients of PNH-AA/MDS and AA with subclinical PNH clones as shown in Table 2. Among classical PNH patients, 28% had ANC < 1500/cu mm and 16.5% had mild thrombocytopenia. This can be explained by the component of bone marrow failure present in classical PNH. This is in concordance with ANC < 1500/cu mm in 21% and thrombocytopenia in 14% of classical PNH patients in a study by Binjen et al. [12]. The mean CRC and the mean LDH was higher in the classical PNH group than PNH/AA.

Data from the International PNH registry reports that 76% of patients with classical PNH but without BMF had clone sizes > 50% and only 8% had clone sizes < 10% [13]. In our study, 96.2% patients of classical PNH had clone size > 50% and none of them had a clone size < 10%. The data on clone sizes in PNH in the context of AA and MDS was variable in the registry with more than 34% of patients having clone sizes of \geq 50% and 40% of patients with lower clone sizes (< 10%) [13]. In our study, a higher proportion of patients (63.9%) in the PNH-AA/MDS group had clone size \geq 50% and only 10% patients had clone size < 20%. A previous study reports a clone size > 20% in 63% of their patients with PNH in the setting of bone marrow failure syndrome as compared to 90% of our patients [14]. A Spanish study reported PNH clones ranging from 2 to 58% with a mean clone size of 10% on the granulocytes in such cases which is much lower than a mean clone size was 65.6% on granulocytes in our study [15].

In the third group of AA/MDS with subclinical PNH clones, 16.2% patients had clone size > 50% with a mean clone size of 22.4% on granulocytes and 24.2% on monocytes. This is in contrast to another Indian study which reported a median clone size of 2.7% on granulocytes and 3.4% on monocytes. They found a clone size > 50% in 9.9% of the bone marrow failure group as compared to 16.2% of our patients of AA/MDS with sub-clinical PNH clones [16, 17]. However, despite a high clone size, none of these patients had elevated reticulocyte count or LDH.

We routinely do not perform testing for PNH clone in MDS patients. However, 42.8% (3/7) patients in whom flow cytometry was done had clone size on granulocytes and monocytes > 20%. 1 of these patients was MDS-SLD-RS and the other two were MDS with multilineage dysplasia (MDS-MLD). None of these patients had any evidence of hemolysis or thrombosis. A previous study reported PNH clone size > 20% in smaller proportion of patients (22%) with MDS or cytopenias and hemolysis [14]. Our observation, however, is in contrast to previous studies which report small PNH clones in MDS patients [14, 15]. Wang et al. in their study showed that the percentage of PNH-type granulocytes varied from 0.003 to 2.41% and was < 1.0% in 17 of 21 (81.0%) PNH patients [18]. We agree that the number of MDS cases in our study is too small, however, it is interesting to note that high PNH clone sizes can be seen in MDS patients. Whether significant multi-lineage dysplasia can be seen in PNH patients or these are PNH patients who progressed to MDS in intriguing.

If we compare the bone marrow cellularity in these 3 groups, 68% of classical PNH patients had hypercellular marrow which is similar to a previous study by Binjen et al. who found a hypercellular marrow in 65% of classical PNH patients [12]. The age-adjusted marrow cellularity was reduced in 91% of PNH/BMF though only 20.6% patients had < 25% marrow cellularity meeting the criteria of severe aplastic anemia. This could be explained because of erythroid hyperplasia in PNH-AA/MDS. All cases of AA with PNH clones had hypocellular marrow whereas all 3 MDS cases with large PNH clones had hypercellular marrow, 2 were hypoplastic and 2 had normocellular marrow. Our findings are similar to a previous study in which 95% of AA-PNH patients and 100% of AA patients had a hypocellular marrow [12].

Although iron stores (grade 0–1 on Perls stain) were reduced in majority of the patients (62.2%) of classical PNH, 37.8% had normal or increased iron stores. This could be possibly because of transfusions or element of bone marrow failure present in all cases of PNH. This is similar to a previous study which reported decreased marrow iron stores in 71% of classical PNH patients. Iron stores were increased in 58% of our PNH/BMF patients and 91% of AA/MDS patients with sub-clinical PNH clones. Similar observations were made by Binjen et al. in their study who described increased iron stores in 64% of AA-PNH patients and 100% of AA patients [12].

We realise that our study has many limitations. The first one is the retrospective nature of the study with no serial follow-up of the patients of PNH-AA and AA/MDS with subclinical PNH clones. Secondly, the number of MDS cases in our study is too small to arrive at statistically valid conclusions. Also, we have selected only patients positive

for PNH clone. So, we do not have data on the prevalence of PNH clones in patients of AA and MDS.

Thus, we conclude that bone marrow failure is the dominant component in PNH patients of Indian ethnic group. However, higher PNH clone sizes are present in Indian PNH-AA and AA/MDS with sub-clinical PNH clones. Though a clone size of > 20–25% on granulocytes and monocytes predisposes the patients to hemolysis, our AA and MDS patients may remain asymptomatic despite presence of large PNH clones. Thrombosis is not an unusual presentation in patients of classical PNH in our setting, though, it is uncommon in cases of PNH-AA. Thrombosis, when present in PNH patients, is associated with high clone size on granulocytes and monocytes.

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Compliance with ethical standards

Conflict of interest None.

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