

Multiparameter FLAER-based flow cytometry for screening of paroxysmal nocturnal hemoglobinuria enhances detection rates in patients with aplastic anemia

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Abstract Flow cytometry is the gold standard methodology for screening of paroxysmal nocturnal hemoglobinuria. In the last few years, proaerolysin conjugated with fluorescein (FLAER) has become an important component of antibody panel used for the detection of paroxysmal nocturnal hemoglobinuria (PNH) clone. This study aimed to compare PNH clone detection by flow cytometry in the pre-FLAER era versus the FLAER era. This was a retrospective analysis of 4 years and included 1004 individuals screened for PNH clone, either presenting as hemolytic anemia or as aplastic anemia. In the pre-FLAER time period, the RBCs and neutrophils were screened with antibodies against CD55 and CD59. With the introduction of FLAER, neutrophils were screened with FLAER/CD24/CD15 and monocytes with FLAER/CD14/CD33 combination. A comparative analysis was done for detection of PNH clone in aplastic anemia patients versus non-aplastic anemia patients, as well as between pre-FLAER and FLAER era. Out of a total of 1004 individuals, 59 (5.8 %) were detected to have PNH clone positivity. The frequency of PNH clone detected in aplastic anemia and non-aplastic anemia groups was 12.02 and 3.36 %, respectively. The detection rate of PNH clone increased from 4.5 % (32/711) in the pre-FLAER era to 9.2 % (27/293) with the introduction of FLAER. However, this increase could be attributed to increased detection of PNH clone in the aplastic

anemia group, which showed a significant increase from 8.3 to 18.2 % after use of FLAER. In the non-aplastic group, PNH clone was detected with similar frequencies before and after use of FLAER (3.2 versus 3.8 %, respectively). Mean PNH clone size was lower in the aplastic anemia group when compared with the non-aplastic group. RBCs always showed a lower clone size than neutrophils. PNH clone on neutrophils and monocytes was however similar. Inclusion of FLAER increases the sensitivity of the test which is especially useful in picking up small PNH clones in patients of aplastic anemia.

Keywords Paroxysmal nocturnal hemoglobinuria · Flow cytometry · FLAER

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematological disorder, caused due to deficiency of membrane-bound glycosylphosphatidylinositol (GPI)-anchored proteins. It is characterized by anemia, intravascular hemolysis, bone marrow hypoplasia, pulmonary hypertension, and tendency to thrombosis [1, 2]. Beside these classical presentations, PNH is known to be associated with various hematological conditions like aplastic anemia and myelodysplastic syndrome [3, 4].

Glycosylphosphatidylinositol-anchored proteins (GPI-AP) are an entire class of cell surface proteins functioning as enzymes, receptors, complement regulators, and adhesion molecules [5]. Deficient GPI-anchored proteins cause increased sensitivity of erythrocytes to complement-mediated cell lysis [2].

Mutation in X-linked gene (Xp22.1) known as phosphatidylinositol glycan class A (PIGA) results in GPI-AP defect [5]. All PNH patients are found to have clonal *PIGA* gene mutations; however, the study by Araten DJ et al.

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showed rare *PIG-A* mutations in the peripheral blood cells of healthy controls, suggesting that *PIG-A* mutations are necessary, but insufficient to cause PNH [6].

Around 70 % of patients of aplastic anemia have a PNH clone detectable by highly sensitive assays, although the underlying mechanism is yet to be unveiled. An attempt to explain the same is by a hypothesis of immune mechanism of selection which states that PNH cells have a proliferative advantage over non-PNH cells [7].

PNH was initially diagnosed using assays like Ham's test and Sucrose hemolysis test [8, 9]. These assays are cumbersome, relatively insensitive, and non-specific as they discriminate PNH cells due to sensitivity to the hemolytic action of complement. These assays have largely been replaced by flow cytometric assays because of the higher sensitivity and specificity when compared to traditional tests, and also that it can accurately measure the size of the PNH cell population. The earlier protocols for screening of PNH clone by flow cytometry required monoclonal antibodies to GPI-anchored proteins like anti-CD59 and anti-CD55 [10]. Furthermore, GPI is receptor for proaerolysin, a bacterial channel-forming toxin derived from *Aeromonas hydrophila*. Proaerolysin was applied in the assay as PNH cells being deficient in GPI were not affected by the toxin [11]. More recently, a modified proaerolysin conjugated with fluorescein (FLAER) was introduced for a flow cytometric diagnosis of PNH. FLAER has the ability to bind to GPI but lacks the channel-forming activity and hence is unable to lyse the cells [12, 13]. A variety of panels comprising of different combinations of monoclonal antibodies with or without FLAER are in use in different laboratories. This study compares the flow cytometric evaluation of PNH clones on CD55 and CD59 expression on neutrophils and RBCs versus use of combination of FLAER and a lineage-associated GPI marker on neutrophils and monocytes.

Material and methods

This retrospective study was carried out at a tertiary care hospital and research institute of north India. The study was conducted over a period of 4 years from July 2008 to August 2012. FLAER was introduced in the PNH screening panel in September 2011, and hence, the study period was split in two time frames, i.e. July 2008 to August 2011 and September 2011 to August 2012. Prior to the use of FLAER, the routine screening panel was composed of antibodies against CD55 and CD59 for analysis of both red blood cells and neutrophils. With the introduction of FLAER, the panel of antibodies was changed to CD15 and CD24 for neutrophils and CD33 and CD14 for monocytes. Routine screening of PNH clone on RBCs was then discontinued. Further, the analysis during each time frame was divided among patients who were

screened for PNH clone after being diagnosed as aplastic anemia on bone marrow examination and the patients who were screened for PNH clone because of other indications, like unexplained hemolytic anemia and thrombosis.

Three-milliliter EDTA-anticoagulated peripheral venous blood sample was collected from each individual. The processing and acquisition of sample was done within 4 h of sampling.

Erythrocyte labeling (direct staining)

Ten-microliter EDTA whole blood was delivered into two BD falcon tubes labeled as negative control, and test. Five microliters each of pre-titrated CD55PE, clone-IA10 and CD59FITC, clone-p282 (H19) (BD Biosciences, USA) were added to the "test" tube, mixed well, and incubated in the dark at room temperature for 30 min. Washing was done with phosphate-buffered saline (PBS), and the cells were resuspended in 500 μ l of PBS [14].

Granulocytes labeling (lyse-stain-wash-no fix technique)

One hundred microliters of whole blood was delivered into two BD falcon tubes labeled as negative control, and test. Two milliliters of RBC lysing solution (prepared in house) was added and kept at room temperature for 10 min. The tubes were centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. The cell button was resuspended in 2 ml PBS, and again centrifuged at 1000 rpm for 5 min. Five microliters of (pre-titrated) CD55PE and CD59FITC were added and incubated in the dark at room temperature for 30 min. This was followed by thorough washing in PBS. Cells were resuspended in 500 μ l of PBS [14].

Following inclusion of FLAER, the processing for screening of granulocytes remained the same, except for antibody panel, which was as follows [15]:

- (i) Neutrophil screening tube: FLAER-AF488/CD24 PE (Clone ML5)/CD15 APC (Clone HI98)
- (ii) Monocyte screening tube: FLAER-AF488/CD14 PE (Clone M5E2)/CD33 APC (Clone P67.6)

The cells were acquired on dual laser BD FACS Canto II. A minimum of hundred thousand events were acquired in each tube. For samples with extremely low leukocyte count, the cells were acquired till exhaustion of the cell suspension. The data was analyzed on BD FACS Diva software.

Based on the data from apparently healthy individuals, the laboratory cutoff for presence of PNH phenotype by lack of expression of CD55 and CD59 was established at 5 %. Therefore, PNH clone sizes below 5 % were not reported during the

initial 3 years of the study period. With the introduction of FLAER, laboratory cutoff value of 0.1 % was achieved both on neutrophils and monocytes.

During the period of use of FLAER, a cocktail comprising of FLAER-AF488/CD55PE/CD15APC was additionally run on peripheral blood samples, using the same processing technique described above, for all individuals detected to have presence of PNH clone. PNH clone on the neutrophils was analyzed and recorded for comparison with results of FLAER/CD24/CD15 combination.

Statistical analysis was done for comparison of PNH clone frequency in the aplastic anemia group versus the non-aplastic anemia group, as well as pre-FLAER versus FLAER groups using chi-square test. Comparison of PNH clone size within the group or inter group comparisons were done using Wilcoxon signed-rank test and Mann Whitney test, respectively. The data analysis for correlation of results using CD55 and CD24 was done using Spearman's rank correlation. All tests were done on SPSS version 22 software.

Results

A total of 1004 individuals were screened for PNH clone by flow cytometry over a time period of 4 years. A total of 59 (5.8 %) patients were detected to have PNH phenotype. Overall, 291 (28.98 %) individuals were diagnosed to have aplastic anemia on peripheral blood and bone marrow examination, and another 713 (71.01 %) individuals were screened for PNH clone for other clinical indications like hemolysis and thrombosis (referred hereafter as the *non-aplastic group*). Among the patients diagnosed with aplastic anemia, 12.02 % (35/291) showed presence of PNH clone, whereas, in the non-aplastic group, PNH clone was noted in 3.36 % (24/713) of individuals. Table 1 summarizes the frequency of PNH clone detection in the two groups, i.e., “aplastic anemia” versus “non-aplastic anemia” clearly shows the higher frequency of PNH clone positivity in the aplastic anemia group.

Seven hundred and eleven individuals were tested prior to introduction of FLAER, whereas 293 were tested with inclusion of FLAER in the screening panel, with frequency of PNH clone positivity of 4.5 % (32/711) and 9.2 % (27/293), respectively. The PNH detection frequency significantly increased

with introduction of FLAER (p value= <0.0001). In the 293 individuals tested for PNH clone using FLAER, 16 individuals had a PNH clone size >5 %. In other words, 5.4 % (16/293) had a clone size significant enough to be detected by using CD55 and CD59 combination alone, which is not significantly different from the detection rate of 4.5 % in the 711 individuals tested without use of FLAER (p value=0.17). Figure 1 shows the gating strategy and depicts a case of aplastic anemia with presence of small PNH clone on neutrophils and monocytes.

Out of 291 patients of aplastic anemia, 181 were screened for PNH clone prior to inclusion of FLAER, using antibodies against CD55 and CD59. PNH clone was diagnosed in 8.3 % (15/181) of the patients. CD55 and CD59 deficiency of >5 % were noted in neutrophils of all 15 patients; however, only 8 patients showed concomitant deficiency on RBCs. Seven patients of aplastic anemia were recently transfused with packed red blood cells and had PNH clone size of <5 % on their RBCs. Table 1S (*Supplementary table*) depicts the flow cytometry results of all individuals positive for presence of PNH clone in both aplastic anemia and non-aplastic groups. The mean percentage of CD55 and CD59 deficiency was 33.9 % (range, 6.3–90.9 %) and 35.1 % (range, 5.2–85.2 %) in neutrophils, respectively. In RBCs, the mean percentage of CD55 deficient was 20.0 % (range, 5.8–67.5 %) and CD59 was 14.1 % (range, 5.3–47.9 %). Out of the 713 individuals of the non-aplastic group, 530 were screened for PNH clone before use of FLAER using antibodies against CD55 and CD59. PNH clone was diagnosed in 3.2 % (17/530). CD55 and CD59 deficiency of >5 % were noted in neutrophils of all 17 patients; however, 16 patients showed concomitant deficiency on RBCs. The mean percentage of CD55 and CD59 deficiency in neutrophils was 62.4 % (range, 35.1–98.5 %) and 58.8 % (range, 15.4–98.1 %), respectively. The mean percentage of RBCs with CD55 and CD59 deficiency were 32.4 % (range, 5.8–58.7 %) and 24.6 % (range, 7.2–53.6 %), respectively.

In the individuals of the aplastic anemia group, neutrophils had significantly larger PNH clone size in comparison to the RBCs, in relation to both CD55 and CD59. Similar result of neutrophils bearing a larger PNH clone size as compared to RBCs was noted in the non-aplastic group also (Table 2).

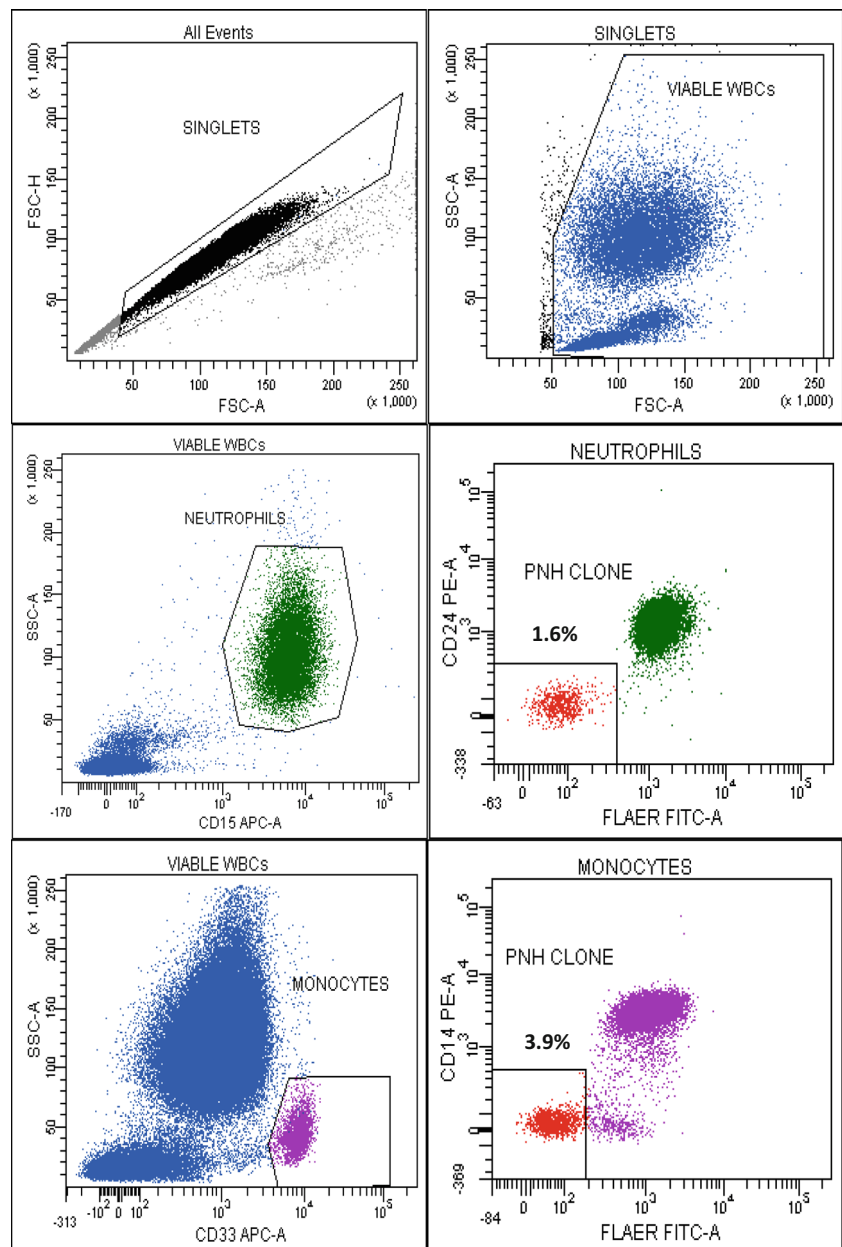
Out of 291 patients in the aplastic anemia group, 110 patients were screened for PNH clone using cocktails of FLAER/CD24/CD15 and FLAER/CD14/CD33 in separate tubes for neutrophils and monocytes, respectively. PNH clone was diagnosed in 18.2 % (20/110) of patients of aplastic anemia. Table 2S (*Supplementary table*) depicts the flow cytometry results of all individuals positive for presence of PNH clone in both aplastic anemia and non-aplastic groups. The mean percentage of FLAER and CD24 (dual) deficient population in neutrophils was 13.3 % (range, 0.6–95.5 %),

Table 1 Comparison of PNH clone positivity in the aplastic anemia group versus non-aplastic anemia group

	Aplastic anemia	Non-aplastic anemia	Total
<i>N</i>	291	713	1004
PNH clone positivity	35 (12.02 %)	24 (3.36 %)	59 (5.8 %)

P value <0.0001

Fig. 1 Scatter plots of cases of aplastic anemia showing presence of small PNH clone. The neutrophils are gated on side scatter (SSC) versus CD15 plot and show 1.6 % CD24- and FLAER-deficient cells. The monocytes are gated on SSC versus CD33 plot and show 3.9 % CD14- and FLAER-deficient cells



and the mean percentage of FLAER and CD14 (dual) deficient population of monocytes was 14.9 % (range, 0.3–84.7 %). Similarly, out of 713 individuals in the non-aplastic group, 183 individuals were screened with the antibody panels inclusive of FLAER. PNH clone was diagnosed in 3.8 %

(7/183) of patients of non-aplastic anemia. The mean percentage of FLAER- and CD24-deficient population in neutrophils was 55.8 % (range, 9.8–91.1 %) and mean FLAER- and CD14-deficient monocytes were 60.7 % (range, 12.4–92.9 %).

Table 2 Comparison of PNH clone size between RBCs and neutrophils in the aplastic anemia and non-aplastic anemia groups

Surface markers	CD55		CD59	
	Aplastic anemia	Non-aplastic	Aplastic anemia	Non-aplastic
Groups	RBCs	Neutrophils	RBCs	Neutrophils
Cell lineage	RBCs	Neutrophils	RBCs	Neutrophils
Mean (% deficient)	20	33.9	14.1	35.1
			24.6	58.8

p value 0.017, <0.001, 0.017, and 0.001

In the individuals of the aplastic anemia group, the PNH clone size was not significantly different among neutrophils ($p=0.376$) as well as monocytes ($p=0.176$), and similar results were noted in the non-aplastic group (Table 3). However, the PNH clone size was much larger among individuals of the non-aplastic group, both on neutrophils ($p=0.002$) and monocytes ($p=0.001$), when compared with the aplastic anemia group.

A combination of FLAER/CD55/CD15 was used on the 20 cases of PNH clone positivity in the aplastic anemia group and 7 positive cases in the non-aplastic group, to compare with the results of FLAER/CD24/CD15 combination on neutrophils. Table 4 compares the PNH clone size of all individuals represented by percentage of neutrophils lacking expression of CD24/FLAER and CD55/FLAER. The mean PNH clone size on neutrophils of all 27 individuals, using FLAER/CD24/CD15 was 24.5 % (range, 0.6–95.5 %) and with FLAER/CD55/CD15 combination was 22.4 % (range, 0.5–98.3 %). There was statistically significant difference in the PNH clone size detected by CD24 when compared to CD55 ($p=0.006$), with a slightly higher mean PNH clone size detected by CD24/FLAER combination. However, in none of the 27 cases, CD55/FLAER combination failed to pick neutrophils with PNH phenotype, and also, there was a significant direct correlation between the PNH clone size measured by using CD24 and CD55 (Spearman's $\rho=0.983$, $p<0.001$).

Discussion

Paroxysmal nocturnal hemoglobinuria is an acquired clonal stem cell disorder with varied clinical manifestations. The traditional laboratory tests for diagnosis of PNH, like Ham's and sucrose lysis tests, relied on in vitro demonstration of increased susceptibility of RBCs to activated complement-mediated lysis. The sensitivity of these conventional tests, especially with small PNH clone, is low, whereas, multiparameter flow cytometry is more specific and quantitative [10]. The ability of flow cytometry to simultaneously study more than one blood cell lineages, multiple GPI-antigens, and directly quantify the clone makes it a standard method to detect PNH clone [16, 17]. This study was spread over a time period encompassing the transition from use of antibodies against

Table 3 Comparison of PNH clone size between neutrophils and monocytes in aplastic anemia and non-aplastic anemia groups

Groups	Aplastic anemia		Non-aplastic	
	Neutrophils	Monocytes	Neutrophils	Monocytes
PNH clone size (%)	13.3	14.9	55.8	60.7

p value 0.376 and 0.176

Table 4 Comparison of PNH clone size on neutrophils of PNH-positive cases ($n=27$) using combinations of FLAER/CD24/CD15 and FLAER/CD55/CD15

S No.	FLAER/CD24/CD15 (%)	FLAER/CD55/CD15 (%)
1	12.5	14.0
2	95.5	98.3
3	1.6	1.5
4	3.1	2.4
5	9.9	7.9
6	1.2	1.2
7	19.4	15.9
8	0.6	0.5
9	12.5	9.2
10	3.3	1.7
11	1.3	1.5
12	40.5	26.1
13	14.6	12.8
14	36.6	28.4
15	1.9	2.2
16	1.2	1.6
17	1.2	0.6
18	1.2	1.5
19	3.1	2.4
20	9.6	9.2
21	74.4	73.7
22	52.0	53.9
23	9.8	8.0
24	29.8	29.2
25	86.5	73.9
26	47.2	40.3
27	91.1	87.4

GPI-anchored proteins alone, to inclusion of FLAER in the panel. Hence, a comparative analysis of data before and after the inclusion of FLAER was attempted. Overall, the frequency of presence of the PNH clone in the aplastic anemia group (29 %) was more than eight times higher than in the non-aplastic group (3.4 %).

The frequency of detecting PNH clone significantly increased from 4.5 to 9.2 % after the use of FLAER ($p<0.0001$). It is also apparent from our results that incorporation of FLAER has primarily enhanced detection rates in the aplastic anemia group, as there has been a rise from 8.3 to 18.2 % in PNH-positive cases in this group, whereas, the frequency of PNH-positive cases remain nearly the same in the non-aplastic group before and after inclusion of FLAER (3.2 and 3.8 %, respectively). In addition, the results also show that, in the group screened for PNH clone using FLAER, the frequency of individuals with >5 % PNH clone has not

changed when compared with the group of individuals screened before use of FLAER ($p=0.17$), clearly indicating the role of FLAER in the increased detection. Studies have shown that at the time of diagnosis of aplastic anemia, approximately one third to one half of cases have elevated numbers of PNH granulocytes; however, the proportion is usually much lower in comparison to classical hemolytic PNH clone size, in which the proportion of erythrocytes and granulocytes that lack GPI-anchored proteins is high, usually above 50 % [7]. Therefore, the increased frequency of PNH clone detection with use of FLAER in our aplastic anemia patients has occurred because of the ability to report PNH clone at a much lower cutoff value of 0.1 %, instead of the cutoff value of 5 % which was used prior to the use of FLAER. There has been increase in sensitivity of PNH clone detection with combination of FLAER with antibody against GPI-anchor protein, like CD24 on neutrophils, and antibody against lineage specific marker for gating of target, like CD15 for neutrophils. This combination has significantly low background positivity in healthy controls, unlike the background noise with use of antibody against CD55 alone. Notably, the study done by Varma N et al., from the same institute, looked into frequency of PNH cells in 90 patients of aplastic anemia, registered during time period of 1996 to 2001. The study reported an incidence of PNH clones of 8.9 % at presentation on flow cytometric analysis with CD55 and CD59 [18]. The frequency of 8.3 % on use of CD55 and CD59, seen in the present study, is comparable to 8.9 % noted in the previous study.

The overall incidence of PNH clone positivity in the present study is about 12 % in patients of aplastic anemia at the time of presentation. This incidence is lower than reported in literature [7, 19]. It is obvious that cases with very small clones of PNH cells, which are not uncommon in patients of aplastic anemia, have been missed in the initial 3 years of study due to higher laboratory cutoff of 5 % in the absence of FLAER. The incidence of 18.2 % seen with use of FLAER in the last year of study is near to that seen by other authors [7, 19]. Importantly, PNH clone detection level in our laboratory has a sensitivity of 0.1 %, whereas, high sensitivity assays in developed nations with dedicated laboratories have PNH clone detection sensitivity of 0.01 %, contributing to higher level of detection. Also, serial evaluations can help increase frequency of detection, whereas, this study evaluated PNH clones only at the time of diagnosis of aplastic anemia. Borowitz et al. (2010) and Sutherland DR et al. (2012) have discussed in great details the guidelines for evaluating PNH clone both on RBCs and granulocytes with a detection level of 0.01 % or even lesser [20, 21]. In case of RBCs, CD55 has been considered inferior to CD59 [21]. In addition, out of the CD59 conjugates tested by Sutherland DR et al., the MEM43 and OV9A2 clones conjugated with phycoerythrin exhibited the best staining characteristics (signal-to-noise ratio) and

allowed optimal separation between Type III and Type II PNH cells as well as separation from Type I (normal) cells while promoting minimal RBC agglutination [21]. Borowitz et al. also used the MEM43 clone of CD59 for high sensitivity assay [20]. Therefore, it is likely that the P282 clone of CD59 used in our study might have contributed to the lack of high sensitivity and thus would have increased the gap in frequency of detection of PNH clone between the “pre-FLAER” versus “FLAER” groups of the present study.

Prior to the use of FLAER, the PNH RBC clone size in both aplastic anemia and non-aplastic groups was significantly smaller than the PNH neutrophil clone. Even in the absence of any history of transfusion, the lower PNH RBC clone in comparison to PNH neutrophil clone is not surprising. The difference in the size of the red cell versus neutrophil PNH clone relates mainly to the fact that red cells remain in the blood for a longer time than neutrophils (e.g., around 3–4 months versus a few days) and therefore they are more prone to suffer direct contact to activated complement proteins and membrane-attack-complexed complement proteins, with cell lysis [22]. However, since assessment of RBCs give better analysis of Type I, Type II, and Type III PNH clones and can be used to monitor increasing clone size in the context of newer therapies such as eculizumab, its use in clinical practice cannot be undermined [22]. In the present study, analysis of CD55 and CD59 expression showed no difference in PNH clone size on neutrophils, whereas, PNH RBC clone size was significantly larger on CD55. The role of CD55, although, has been debated previously, due to its lower sensitivity and specificity, and also sub-optimal division of RBCs into Type I, Type II, and Type III PNH phenotypes as compared to CD59 [23].

The PNH neutrophil and PNH monocyte clones analyzed with a cocktail of antibodies along with FLAER did not show any significant difference of clone size in the two cell lineages, in both aplastic anemia and non-aplastic groups. However, the comparison of the clone in the aplastic group with the non-aplastic group showed that both neutrophils and monocytes had a significantly larger PNH clone in the non-aplastic group. This is consistent with previously published studies which state that PNH clone size is usually small especially at presentation of aplastic anemia as compared to individuals presenting with hemolytic or thrombotic episodes [7].

The present study also compared efficacy of antibodies against CD55 with CD24 for their ability to detect PNH neutrophils in 27 individuals. The mean clone size of PNH neutrophils was significantly higher with use of CD24 (24.5 %) as compared to CD55 (22.4 %); however, the sensitivity of FLAER/CD55/CD15 combination was good enough to detect PNH neutrophils in all the 27 cases (Table 4). In addition, the PNH clone size detected by FLAER/CD55/CD15 combination directly correlated with that detected using FLAER/CD24/CD15 combination. Olteanu H et al. had

shown equal efficacy of CD55 and CD24 for detecting PNH neutrophils in their cohort of 15 patients [24]. Hence, although CD24 showed a better sensitivity in our cohort, it is evident that antibody against CD55 may be used as an alternative for screening of PNH clone on neutrophils.

We compared the data of individuals screened for PNH clone positivity with traditional methods of using CD55 and CD59 to detect PNH clone in RBC and neutrophil lineages with data of separate group of individuals screened with multiparameter FLAER-based assay for PNH clone detection in monocyte and neutrophil lineages, each group comprising of individuals screened for PNH clone upon diagnosis of aplastic anemia and individuals screened for PNH clone due to hemolytic or thrombotic presentation (non-aplastic group). Our data demonstrates superiority of the multiparameter FLAER assay over the traditional assay in individuals with aplastic anemia, which showed at least a twofold rise in frequency of detection with FLAER assay. The two methodologies were equally efficient in the non-aplastic group which unlike aplastic anemia, usually do not present with very small clone sizes.

Conclusion

Multiparameter FLAER-based assay is a more sensitive assay in comparison to traditional CD55- and CD59-based assay, for detection of PNH clone in patients of aplastic anemia. The introduction of FLAER in screening assay produced a twofold rise in PNH clone detection frequency. The efficacy of the two assays was comparable in cases of hemolytic or thrombotic PNH.

Conflict of interest The authors declare that they have no conflict of interest.

Authors' contribution Man Updesh Singh Sachdeva contributed in the compilation of data, statistical analysis, and writing of the manuscript. Neelam Varma formulated the study design and edited the manuscript to its final shape. Dinesh Chandra collected the data and helped in the writing of the manuscript. Praveen Bose carried out flow cytometry for evaluation of PNH clone. Pankaj Malhotra and Subhash Varma were the treating physicians and have provided clinical details of the patients.

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