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ORIGINAL ARTICLE

FLAER as a standalone reagent for paroxysmal nocturnal hemoglobinuria: Do we need to reconsider the guidelines for testing?

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Abstract

Introduction: Flow cytometry-based paroxysmal nocturnal hemoglobinuria (PNH) testing involves utilization of monoclonal antibodies against GPI-linked proteins and FLAER. The ability of FLAER to bind to a wide variety of GPI-linked structures and to be utilized across different leukocyte subsets is remarkable. We hypothesize that FLAER as a standalone reagent may be equally effective for detecting PNH clones. The present study intends to compare the results of a FLAER alone-based strategy to the recommended FLAER+GPI-linked protein-based approach for applicability in clinical settings.

Methods: EDTA-anticoagulated blood samples from patients for PNH workup were tested for PNH by multiparametric flow cytometry. A conventional panel comprising gating markers (CD45 for WBC, CD15 for granulocytes, and CD64 for monocytes) and a combination of FLAER and GPI-linked markers, such as CD24 and CD14, henceforth referred to as the "routine panel," was employed. Second, a "FLAER-only panel" comprising the gating markers and FLAER alone (excluding the GPI-linked markers CD24 and CD14) was set up. The samples were processed using the lyse-wash-stain-wash technique, and events were acquired on BC Navios Ex flow cytometer (Beckman Coulter, Inc., USA) and analyzed on Kaluza Software 2.1. The presence of a PNH clone was reported at a value of ≥0.01%.

Results: A total of 209 patients were tested. Both panels found a PNH clone in 20.1% of patients ($n = 42/209$) with a 100% concordance rate. The PNH clone range for granulocytes was 0.01%–89.68%, and for monocyte was 0.04%–96.09% in the *routine panel*. The range in the *FLAER-only panel* for granulocytes was 0.01%–89.61%, and for monocytes, it was 0.01%–96.05%. Pearson correlation statistics revealed a significant correlation between the size of the PNH clone of granulocytes and monocytes among the two panels tested (granulocytes $r = 0.9999$, $p < 0.0001$, 95% CI $= 0.9999$ to 1.000; monocytes $r = 0.9974$, $p < 0.0001$, 95% CI $= 0.9966$–0.9980).

Conclusion: Based on our results, FLAER as a standalone marker is specific and sensitive for identifying PNH clones in granulocytes and monocytes, even for

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high-sensitivity PNH assay. The proposed "FLAER-only panel" panel is efficient and cost-effective for highly sensitive PNH testing in two different cell lineages, especially in resource-limited clinical settings.

KEYWORDS
CD55, CD59, FLAER, flow cytometry, PNH

1 | INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare non-malignant hematological disease. The defect in PNH is genetically defined by somatic mutations of the phosphatidyl-inositol glycan (PIGA) gene in the hematopoietic stem cells (HSC), leading to the clonal expansion of abnormal HSCs.[1–3] Consequent to the mutations in the *PIGA* gene, there is the loss of glycosylphosphatidylinositol (GPI)-anchored proteins from the cell surface[4,5] such as CD55 (decay accelerating factor) and CD59 (membrane inhibitor of reactive lysis), leading to increased sensitivity of red blood cells (RBCs) to complement-mediated lysis.[6,7]

Despite PNH being described as early as 1882,[8] the majority of progress in understanding disease biology, pathogenesis, diagnosis, and intervention has occurred in the last three decades.[9–11] The diagnosis of PNH has now become straightforward with the advent of flow cytometry, where cell populations deficient in GPI-anchor-related proteins can be readily identified by targeted monoclonal antibodies on peripheral blood testing.[12–14] Traditionally, most flow cytometric PNH approaches aimed at demonstrating the lack of complement regulatory proteins such as CD55 and CD59 in the RBC and later in leukocytes. Monoclonal antibodies to various GPI-anchored markers, such as CD24, CD14, and CD157 became increasingly available for PNH testing on different hematopoietic subsets. The specific issues related to monoclonal antibodies to GPI-linked proteins are of concern and need to be addressed. The expression of CD14 is often low on CD64^{high}/CD15^{low} monocytes visualized as a trailing effect on FLAER versus CD14 plot creating confusion in interpretation and with potential for false positive labeling as PNH clone.[15,16] Moreover, in clinical conditions such as myelodysplastic syndromes, monocytes weakly express CD15, or monocytes are gated with dysplastic neutrophils with decreased granularity resulting in false negative CD24 events in the gated population.[15] The expression of CD157 is low on the eosinophils, which might confuse when CD15+ neutrophils are imprecisely gated.[17] Also, certain genetic or ethnic variants of CD157 may lead to false-positive PNH clone identification.[18,19] Recommendations for PNH requires testing at least two GPI-anchor proteins using two different monoclonal antibodies on more than one cell lineage for an unequivocal diagnosis of PNH.[14,20–22] The introduction of a flow cytometric assay based on FLAER (fluorescently labeled inactive variant of the protein aerolysin) has improved the accuracy and sensitivity of PNH clones.[1,22–25] In contrast to the fluorochrome labeled monoclonal antibodies, which bind to the GPI-linked proteins, FLAER (conjugated to Alexa488) has a remarkable specificity for the GPI anchor itself. FLAER has been helpful in detecting PNH clones on WBCs but not on erythrocytes and the role of FLAER in the efficient detection of PNH clones is established compared to non-FLAER based flow-approaches. The first-of-its-kind published guidelines from the International Clinical Cytometry Society (ICCS)[26] retained the recommendation of using two reagents (one should be FLAER) for high-sensitivity PNH testing across two different cell lineages, further endorsed by the 2012 Practical Guidelines[27] and 2018 ICCS/ESCCA consensus groups.[28–31] The latest 2018 ICCS/ESCCA consensus, describing four-, five- and six-color antibody panels[28–31] had different combinations of monoclonal antibodies to GPI-linked proteins; however, FLAER was consistently deployed in each of the proposed antibody panels.[29]

With documented pitfalls in interpreting flow-based PNH testing using antibodies against GPI-anchored proteins, it has been suggested to consider the possibility of using FLAER alone based strategy for PNH testing on WBCs.[16] In the present study, we hypothesize and aimed that FLAER as a standalone reagent may be equally effective for detecting PNH clones. We compared the validity of a FLAER only based strategy to the conventional FLAER+GPI-anchored marker-based approach for applicability in clinical settings.

2 | PATIENTS AND METHODS

This study was conducted in the Department of Hematology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. Peripheral blood (PB) samples from patients for PNH workup received in the flow cytometry laboratory at the Department of Hematology were tested for PNH by multiparametric flow cytometry. The Institutional Ethical Clearance Committee (IEC) approval was obtained for the conduct of the study. All procedures performed in the present study were by the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments (2008).

2.1 | Monoclonal antibody (mAB) panels for the detection of PNH

"Routine panel": This comprised of gating markers CD45, CD15 (for neutrophils) and CD64 (for monocytes), and a combination of FLAER along with GPI-linked markers CD24 and CD14 for neutrophils and monocytes respectively. "FLAER only Panel": This comprised of the gating markers (same as those in routine panel) and

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TABLE 1 The details of the PNH assay panel used.

	CD45	CD15	CD64	CD24	CD14	FLAER
Routine panel	+	+	+	+	+	+
FLAER only panel	+	+	+	−	−	+
GPI-status	Non-GPI-linked	Non-GPI-linked	Non-GPI-linked	GPI-linked	GPI-linked	GPI-linked
Purpose	Gating of WBCs	Gating of neutrophils	Gating of monocytes	Detecting PNH clone in neutrophils	Detecting PNH clone in monocytes	Detecting PNH clone in neutrophils and monocytes
Fluorochrome	KrO	PB	APC750	PC5.5	APC	Alexa488
Clone	J33	80H5	22	ALB9	RMO52	NA
Titration	5 μL	10 μL	5 μL	5 μL	3 μL	5 μL
Vendor	Beckman Coulter, USA	Beckman Coulter, USA	Beckman Coulter, USA	Beckman Coulter, USA	Beckman Coulter, USA	Cedarlane, Victoria, BC, Canada

FLAER (excluding the GPI-anchored markers CD24 and CD14). The details of monoclonal antibodies used in this study, as shown in Table 1.

2.2 | Sample processing and acquisition

Two to three milliliter of EDTA anticoagulated venous PB sample was collected in lavender-topped vacutainers (Becton Dickinson, CA, USA) and the samples were processed within 24 h of collection. Pre-titrated antibody cocktails were used. A volume of 100 μL sample was taken (more in cytopenic patients) and processed using lyse-wash-stain-wash technique. RBCs were lysed using an ammonium chloride-based lysing agent (containing ethylenediamine tetraacetic acid—0.037 g, NH_4Cl—8.26 g, $KHCO_3$—1 g in 1000 mL de-ionized water, pH 7.2–7.4) as described previously.[25,32] After appropriate washing with phosphate-buffered saline containing 0.1% bovine serum albumin, the cell suspension was incubated with pre-titrated FLAER and fluorochrome-conjugated cocktail monoclonal antibodies in two separate tubes, that is, routine panel tube and FLAER-only panel tube, as mentioned in Table 1. After final washing, the samples were run (within 30 min) on Navios EX flow cytometer (Beckman Coulter, Inc., USA). Atleast 100 000 neutrophils and 20 000 monocytes were acquired for assessment of PNH clone on neutrophils and monocytes, respectively. Cytopenic samples were run until exhausted and maximum possible events were acquired. Analysis was performed using Kaluza software (v2.1) (Beckman Coulter, Inc., USA). Ten normal controls were studied for the determination of background event levels. A fresh PNH sample under serial dilutions of 1:10, 1:100, 1:1000, 1:10 000, and 1:100 000 (vol:vol) with a normal blood sample was stained with both the panels for determination of assay sensitivity. PNH clone was reported with a cluster of at least 30 neutrophil events and at least 15 monocyte events in the FLAER-negative (and CD14/CD24 negative-in routine panel) quadrant. As per the current terminology, a more appropriate term, "neutrophils," has been used over "granulocytes."

2.3 | Statistical methods

Descriptive analysis has been presented as median and range. The Gaussian distribution of data was checked using the Shapiro–Wilk test. For normally distributed datasets, paired Student's *t*-test was used to compare two groups, while the Mann–Whitney *U* test was performed for non-normally distributed data. Pearson correlation analysis was performed in FLAER, CD24, and CD14 markers. All statistical tests we used in this study were two-tailed, with a significance level of $p < 0.05$ (represented as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). All the statistical analyses were performed using licensed GraphPad Prism (v9.2).

3 | RESULTS

3.1 | Patient cohort and demographic characteristics

A total of 209 patients were included in the study. Of 209 patients tested, in 161/209 (77.03%) patients, two tubes (Routine and FLAER only) were run in parallel. The PNH clone was determined in both tubes separately. In 47/209 (22.4%) patients, only the routine tube was run where the PNH clone was determined in the same tube using CD24 versus FLAER, CD14 versus FLAER combination (equivalent to the routine panel), and FLAER only-histograms or FLAER versus negative channel (equivalent to FLAER only panel).

The mean age of the patients was 46 years (22–75 years), and that of the controls was 50 (25–69 years). There was no significant difference in the mean age between the two groups. The male: female ratio amongst the patient groups was 1.8:1.

3.2 | Background PNH phenotypes in normal controls ($N = 10$)

We analyzed 10 normal controls to determine the background event rate using both "routine panel" and "FLAER-only panel." Among these

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normal samples, using the "FLAER-only panel", a mean of 535 424 neutrophil events were collected (range 129 129–977 404) and a mean of 55 902 monocyte events were collected (range 26 958–107 602). The background event rate in neutrophils was a mean of 0.5 events (range 0–2) and for monocytes were 0. Further, using the "routine panel," a mean of 638 068 neutrophil events were collected (range 135 831–1 052 271) and a mean of 68 029 monocyte events were collected (range 39 216–124 031). The background event rate in neutrophils was a mean of 1.2 events (range 0–3) and for monocytes was 0.1 (0–1).

TABLE 2 Assay sensitivities of FLAER-only and routine panel determined using spiking experiment.

Sample dilution	Neutrophil assay	Monocyte assay
FLAER-only panel assay sensitivity		
1:10	4.96	14.53
1:100	0.46	1.63
1:1000	0.05	0.24
1:10 000	0.007	0.029
1:100 000	0.002	0.006
Routine panel assay sensitivity		
1:10	6.06	15.743
1:100	0.59	1.637
1:1000	0.063	0.19
1:10 000	0.009	0.02
1:100 000	0.001	0.004

3.3 | Assay sensitivities of "FLAER-only panel" and "routine panel"

A fresh sample positive for a major PNH clone (~98.12% granulocyte PNH clone and ~98.74% monocyte PNH clone size) was serially spiked 1:10 to 1:100 000 with a normal sample using published strategies (Available as Supporting Information).[27] The lower limit of quantification (LLOQ) and limit of detection (LOD) were determined (Table 2). The LLOQ and LOD for neutrophils using the FLAER-only panel was 0.007 and 0.002, for monocytes was 0.24 and 0.02, respectively. The LLOQ and LOD for neutrophils using the routine panel was 0.009 and 0.001, for monocytes was 0.19 and 0.02, respectively.

3.4 | Comparison of results of both the panels ($N = 209$)

A PNH clone was found in 42/209 patients (20.1%) by both the panels tested. The PNH clone range for neutrophils was 0.01%–89.68%, and for monocyte was 0.01%–96.09% in the routine panel. The range in the proposed panel for neutrophils was 0.01%–89.61%, and for monocyte, it was 0.01%–96.05%. The gating strategy using

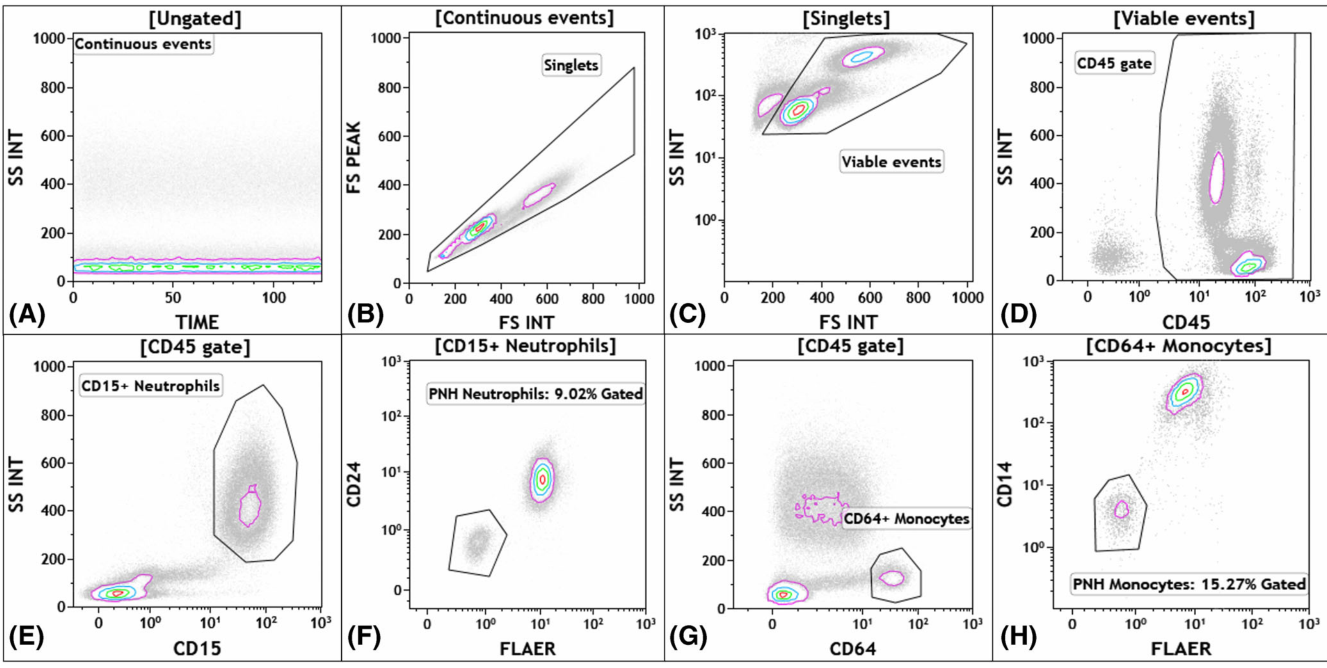

FIGURE 1 (A–F) Flow cytometric dot plots from the "routine tube." (A) The continuous events are gated on SSC versus TIME plot, and these are further gated on (B) FS PEAK versus FS INT for doublet discrimination. (C) The gated singlet events are plotted SS INT versus FSC INT for exclusion of cellular debris. (D) The CD45 plot for WBC distribution is subsequently studied on (E) SSC versus CD15 plot for gating of neutrophils. (F) The neutrophils are plotted on CD24 versus FLAER. FLAER−/CD24− (double negative) population constitute the PNH neutrophil clone (9.02% of all gated neutrophils). (G) Similarly, the monocytes are gated on an SSC versus CD64 plot. (H) The PNH monocytes are FLAER−/CD14− (double negative) events (15.27% of all gated monocytes).

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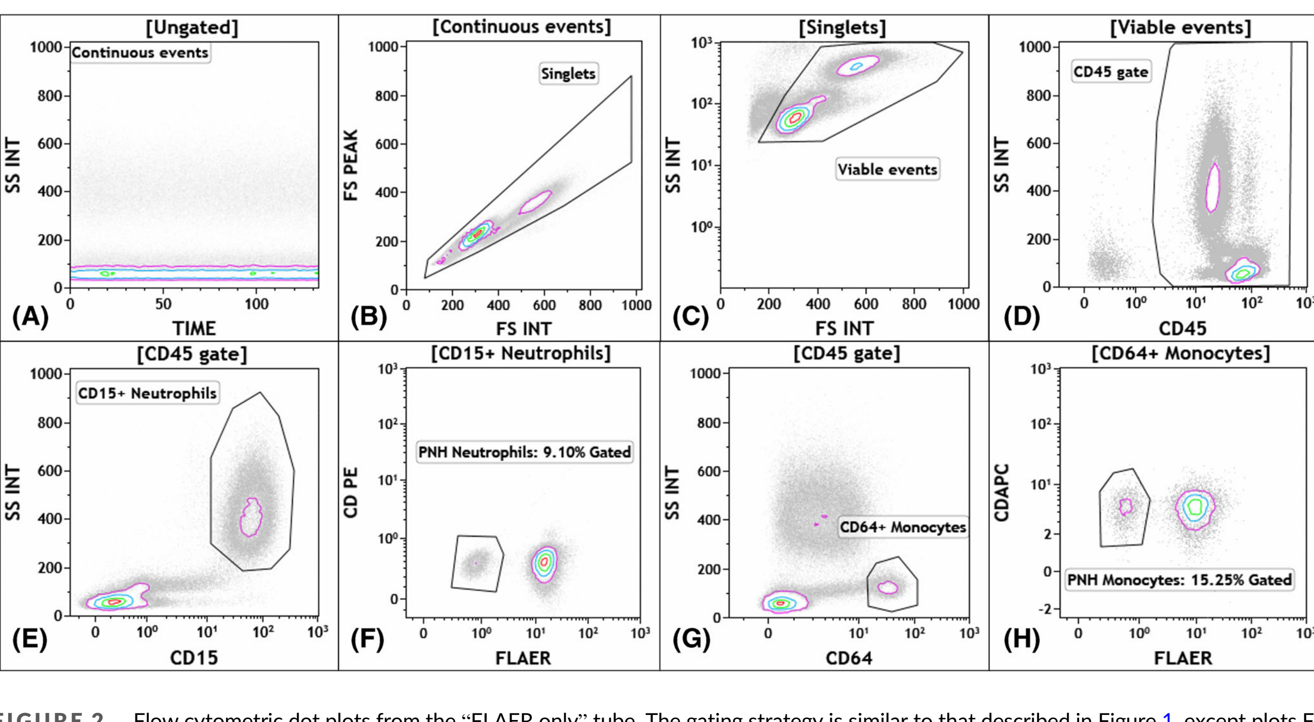

FIGURE 2 Flow cytometric dot plots from the "FLAER only" tube. The gating strategy is similar to that described in Figure 1, except plots F and H. As CD24 and CD14 are excluded in the FLAER only tube, FLAER is plotted against empty fluorochrome channels. FLAER histograms are an alternative, however, the strategy mentioned above was more appropriate.

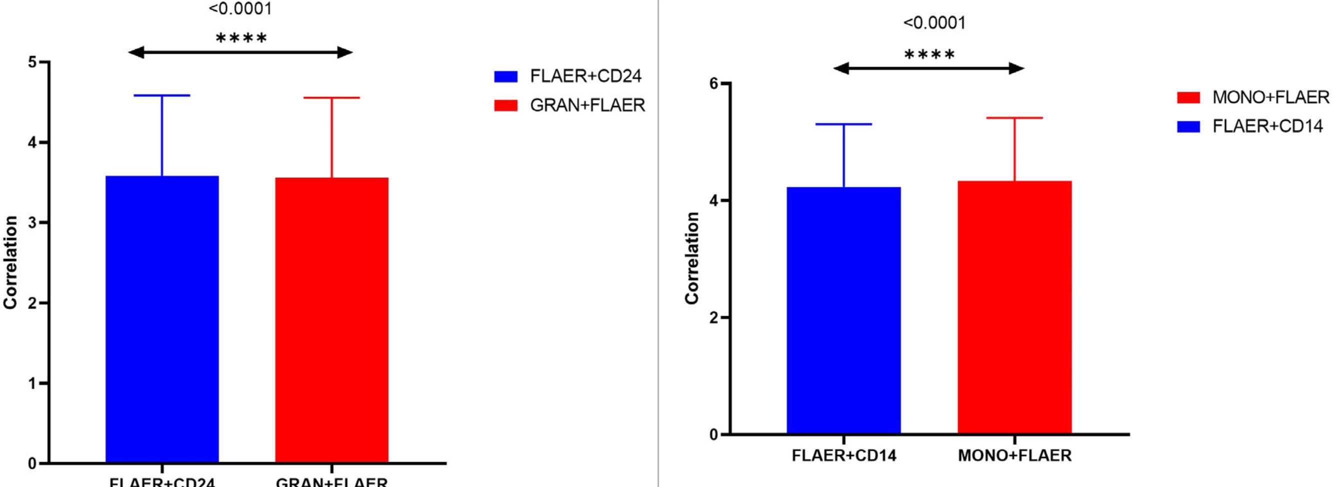

FIGURE 3 Pearson correlation statistics of results of both the tubes ("routine tube" has been labeled as FLAER+CD24 and FLAER+CD14, and "FLAER-only tube" as GRAN+FLAER and MONO+FLAER). The left image shows correlation between the PNH results of neutrophils and the right image is of the monocytes.

both panels is shown in Figures 1 and 2. There was a significant correlation between the size of the PNH clone of neutrophils and monocytes among the two panels (neutrophils $r = 0.9999$, 95% confidence interval = 0.9999 = 1.000, $p < 0.0001$; monocytes $r = 0.9974$, 95% confidence interval = 0.9966–0.9980, $p < 0.0001$). Figure 3 shows the correlation statistics between the panels on neutrophils and monocytes. Also, on paired test analysis, there was no statistical difference between the two tubes for identifying PNH (neutrophils $p = 0.62$, monocytes $p = 0.21$).

4 | DISCUSSION

Flow cytometric PNH clone identification relies on the expression of GPI-linked markers on blood cell types. While monoclonal antibodies directed against various GPI-linked anchor proteins are commonly used, FLAER is a particularly useful reagent that binds to the GPI anchor itself. The addition of FLAER increased the sensitivity and accuracy of the PNH assay.[1,22,23,26,29,33] We had previously compared the non-FLAER based versus FLAER-based PNH clone detection rate

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and found an increased sensitivity of the flow-based FLAER approach for picking up minor PNH clones in patients with aplastic anemia.[32]

FLAER is highly specific to bind to the GPI anchor itself, enabling a more accurate assessment of the PNH clone. It lacks deficiencies associated with the monoclonal antibodies, and the available literature does not cite any direct disadvantage of FLAER in the evaluation of PNH clones compared to monoclonal antibodies. There are some advantages in the proposed FLAER-only tube apart from achieving high sensitivity equivalent to the routine panel. It is known that increasing the number of fluorochrome-labeled antibodies in a panel increases the complexity of an assay with high chances of non-specific binding, cell aggregation, and florescent spillover to the adjacent filters leading to difficulties in assay standardization and validation.[34,35] Lesser antibodies in the FLAER-only tube may overcome these challenges, simplifying the assay. In addition, there is a reduction in the cost of testing for the laboratory, with at least a 20 USD difference in using a FLAER-only panel versus the routine panel in our settings. In a centrally funded, government-subsidized facility like ours, adopting this approach results in an annual cost savings of around 10 000 USD (500 tests/year). It is important to note that private laboratories may experience even greater cost savings when implementing this strategy. Further, we identified internal controls using both panels, as internal controls can ensure appropriate instrument voltage and compensation settings. Lymphocytes are not a suitable target for the quantification of PNH clones as they are long-lived. However, they serve as good internal controls.[29] In the case of a routine panel, while testing the lymphocytes on FLAER versus CD24 combination, the presence of FLAER+/CD24+ B-cells verifies that both the reagents were added, NK- and T-cell subsets can be identified, which are FLAER+/CD24−. Similarly, in the FLAER-only tube, FLAER+ lymphocytes can serve as an internal control.

The current and previous consensus group recommendations and guidelines require testing for at-least two GPI-linked structures on at least two or more blood cell lineages.[26,27,29] These recommendations can be traced to the pre-FLAER era, where moAbs were primarily used for PNH diagnosis.[14,20,21] The use of moAbs has always been a concern as no single antibody was optimal for testing a GPI-anchor defect on all cell lineages.[22] FLAER is superior to moAbs as the binding of FLAER significantly represents the expression of GPI-anchor much more than moAbs.[22] The use of FLAER-only as a stand-alone reagent and completely abandoning GPI-markers other than FLAER for testing of PNH has already been suggested.[16]

The use of a FLAER-only panel has a few restrictions. The distinction of Type II, Type III, and normal populations of neutrophils and monocytes using FLAER alone histograms or dot plots might not be easier due to challenges in setting up precise gates. While dual-parameter dot plots perform better in distinguishing these populations, it might still be challenging.[30] The current assay exhibits certain limitations, which are consistent across both the "routine panel" and "FLAER-only panel" strategies. These limitations, although present, do not impact the conclusions of our study but warrant acknowledgment. The practical guidelines and ICCS consensus recommendations advocate the stain-lyse-wash technique, with lysing agents containing fixatives being preferred due to their ability to minimize continuous neutrophil degranulation and interference in the assay.[27,29] In our laboratory, we have successfully employed the lyse-wash-stain-wash technique using an ammonium-chloride-based lysing agent without fixative, yielding positive results.[25,32] Also, the antibody-fluorochrome combination employed in this study deviates from the recommended international consensus guidelines. It is suggested to consider conducting a subsequent study with antibodies that adheres to the consensus panel's recommendations.

In the present study, we have demonstrated an excellent correlation between the routine and FLAER-only panels for the identification of PNH clones in clinical samples. Although the approach based on two GPI-linked marker analyses per blood cell lineage is recommended to increase the robustness and accuracy of the PNH assay, we have demonstrated that FLAER as a single reagent is sufficient for the assessment of PNH clone on two cell lineages simultaneously. FLAER, as a standalone marker, in our experience, is specific and sensitive for the identification of PNH clones in neutrophils and monocytes. The proposed "FLAER-only" panel is efficient and cost-effective for highly sensitive PNH testing, especially in resource-limited settings. The study is a pilot work for establishing the hypothesis, and multi-centric studies with rigorously standardized approaches must be performed for successful implementation in clinical practice.

AUTHOR CONTRIBUTIONS

Praveen Sharma, Man Updesh Singh Sachdeva: Conceptualization. **Suneel Rachagiri, Arun Kumar, Jasbir Kaur, Parveen Bose:** Methodology. **Praveen Sharma, Parveen Bose, Dikshat Gopal Gupta, Nabhajit Mallik:** Formal analysis and investigation. **Praveen Sharma:** Writing—original draft preparation. **Man Updesh Singh Sachdeva, Neelam Varma, Pankaj Malhotra:** Writing—review and editing.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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