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FLAER Revealed Normally Expected Non-PNH FLAER-Dim Immature Myeloid Cells (CD117+/CD34-) In Bone Marrow Aspirates and Could Be Utilized as a Marker of Hierarchical Hematopoiesis

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ABSTRACT

Introduction: Fluorescently labeled aerolysin (FLAER) is widely used for the identification of paroxysmal nocturnal hemoglobinuria (PNH) clones in peripheral blood (PB) samples. However, there are only a few reports on the differential fluorescent intensity of FLAER in normal bone marrow (BM) cell subpopulations. The purpose of this study was to evaluate FLAER expression during normal and pathological hematopoiesis, to map the critical existence of non-PNH FLAER-dim cells.

Methods: A total of 54 BM aspirates were prospectively analyzed with FLAER-based flow cytometric (FC) protocols, during their routine work-up. These were obtained from patients with the following diagnoses: PNH (3), infections/reactive (5), myelodysplastic syndromes (MDS) (7), myelodysplastic/myeloproliferative neoplasms (MDS/MPN) (4), chronic myelogenous leukemia (CML) (3), acute myelogenous leukemia (AML) at diagnosis (20), AML in measurable residual disease (MRD) assessment (7), and B-cell acute lymphoblastic leukemia (B-ALL) in MRD assessment (5). The applied protocols consisted of FLAER, HLA-DR, CD14, CD33, CD34, CD66b, CD38, CD117, CD64, CD45, and FLAER, CD66c, CD14, CD33, CD34, CD66b, CD123, CD16, CD64, and CD45, respectively. FLAER expression was assessed in CD34++/CD38- and CD34+/CD38+ stem cells, CD34-/CD117+/HLA-DR+/CD33+ myeloid precursors, and CD64+/CD14-/HLA-DR+ monocyte precursors but also in mature myeloid cells.

Results: All patients revealed an intermediate FLAER intensity in CD34++/CD38- stem cells, with a discrete FLAER-negative subpopulation observed only in maturing CD34+/CD38+ stem cells of patients with PNH. The lowest FLAER intensity was noticed in CD34-/CD117+/HLA-DR+/CD33+ myeloid precursors, not only in patients with PNH but also in PNH-negative BM aspirates. An ascending FLAER intensity was further observed during monocytic and granulocytic maturation, with a discrete FLAER-negative population in CD64+/CD14-/HLA-DR+ monocyte precursors and maturing neutrophils and monocytes of patients with PNH only. The maturation pattern of FLAER expression was further confirmed in a patient with acute promyelocytic leukemia treated with all-trans retinoic acid (ATRA), where FLAER was concurrently upregulated with CD66b in a consecutive series of PB samples tested over a 20-day-period after diagnosis.

Conclusion: The application of FLAER in PNH-positive and PNH-negative reactive or malignant BM aspirates identified normally expected non-PNH FLAER-dim CD34-/CD117+/HLA-DR+/CD33+ myeloid precursors in all samples. A specific FLAER-associated maturation pattern was observed, which is proposed for further study within MRD and diagnostic protocols.

1 | Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is unique because it is an acquired hemolytic anemia, resulting from an intrinsic red cell membrane disorder [1]. The disease has been shown to be due to a somatic mutation of the phosphatidylinositol glycan complementation class A (PIG-A) gene at the level of the hemopoietic stem cell [1]. The defect in the synthesis of the glycosylphosphatidylinositol (GPI) anchor results in a deficiency of all proteins that are GPI-bound to red cell, leucocyte, and platelet membranes [1].

Flow cytometry (FC) has become the "gold standard" for detecting abnormal clones in PNH, aplastic anemia (AA), and myelodysplastic syndromes (MDS) [2]. It uses combinations of lineage-defining antibodies, in conjunction with monoclonal antibodies against specific GPI-anchored proteins such as CD55, CD59, or with fluorescently labeled aerolysin (FLAER), a mutant variant of the bacterial toxin aerolysin that binds to GPI molecules [3]. PNH cells show poor expression of surface proteins such as CD55 and CD59, and dim or absent binding of FLAER. Thus, a PNH clone is defined as the fraction of cells demonstrating decreased or absent GPI-anchored antigens or FLAER binding [4].

Detection of PNH clones in peripheral blood (PB) with FLAER and antibodies to GPI-linked proteins is a well-established and standardized approach. In contrast, bone marrow (BM) specimens are generally considered less suitable than blood owing to variable expression of GPI-linked antigens during hematopoietic cell differentiation [4]. A similar approach in BM would encounter difficulties, because in non-PNH hematopoiesis, there is a reduced expression of GPI proteins such as CD14, CD66b, and CD16, which are usually used in routine FC protocols to assess immaturity and differentiation. The purpose of this study was to compare FLAER expression in several BM subpopulations when applied in normal and pathological BM aspirates, in the presence or absence of a PNH diagnosis.

2 | Materials and Methods

The study aimed to compare the pattern of FLAER expression between patients that had been previously diagnosed with PNH from PB samples and patients with a known hematological diagnosis or nonmalignant subjects (reactive BM aspirates). FLAER expression in immature and mature cell subpopulations of BM aspirates was compared between PNH and non-PNH subjects. More specifically, 54 BM aspirates were prospectively analyzed with FLAER-based protocols, during their routine work-up. These were obtained from patients with the following diagnoses: PNH ($n=3$), infections/reactive ($n=5$) (reactive category), myelodysplastic syndromes (MDS category) ($n=7$), myelodysplastic/myeloproliferative neoplasms (MDS/MPN category) ($n=4$), chronic myelogenous leukemia (CML or MPN category) ($n=3$), acute myelogenous leukemia (AML) at diagnosis ($n=20$) (AML-Dx category), AML in measurable residual disease (MRD) assessment ($n=7$) (AML-MRD category), and B-cell acute lymphoblastic leukemia (B-ALL) in MRD assessment ($n=5$) (B-ALL-MRD category). The FLAER-based protocols were applied as additional protocols, in the context of their routine work-up. Cases that were tested for the presence of MRD were found negative. Also, all PNH-negative patients were recruited after testing negative in the investigation for a PNH clone in PB. An informed consent was obtained from all patients recruited in the study.

Two FC protocols were prospectively applied in all BM aspirates. These consisted of FLAER(-FITC), HLA-DR(-PE), CD14(-ECD), CD33(-PC5.5), CD34(-PC7), CD66b(-APC), CD38(-A700), CD117(-A750), CD64(PB), CD45(K) and FLAER(-FITC), CD66c(-PE), CD14(-ECD), CD33(-PC5.5), CD34(-PC7), CD66b(-APC), CD123(-A700), CD16(-A750), CD64(-PB), and CD45(-K), respectively. The hierarchical study discriminated CD34++/CD38- as ancestral CD34+ cells, CD34+/CD38+ stem cells as more mature stem cells, CD34-/CD117+/HLA-DR+/CD33+ myeloid precursors, and CD64+/CD14-/HLA-DR+ monocyte precursors. FLAER expression was assessed in these immature subpopulations but also in mature subpopulations, including neutrophils and monocytes. The gating strategy, which was applied for the investigation of CD34++/CD38- stem cells, CD34+/CD38+ stem cells, and CD34-/CD117+/HLA-DR+/CD33+ myeloid precursors, is described in the Figure S1. The respective gating strategy that was followed for the investigation of immature and mature monocytes is described in Figure S2. Mast cells, plasma cells, and NK cells were also evaluated (data not shown). FLAER highest intensity was defined by normal polymorphonuclear neutrophils (PMN) and minimal intensity by PNH-PMN. Special care was provided to FLAER titration and compensation. All fluorochrome-conjugated monoclonal antibodies were obtained from Beckman Coulter (Miami, FL, USA) and were used in the amount of 5 μL in each protocol, except FLAER that was used in the amount of 10 μL. All samples were analyzed, using a Navios flow cytometer (Beckman Coulter; Miami, FL, USA).

3 | Results

3.1 | Patients With PNH

A qualitative identification of the four cell populations of interest (as described in methods) in patients with PNH, is depicted in Figure 1 (upper plots), in Figure 2 (left plots) and in Figure 3 (upper plots). Patients with PNH appeared with an obvious existence of FLAER-negative cell populations (Figure 1A). In CD34+ cells, an obvious FLAER-negative subpopulation was observed (Figure 1B–D), mainly in more mature CD34+/CD38+ cells (Figure 1D). At the same time,

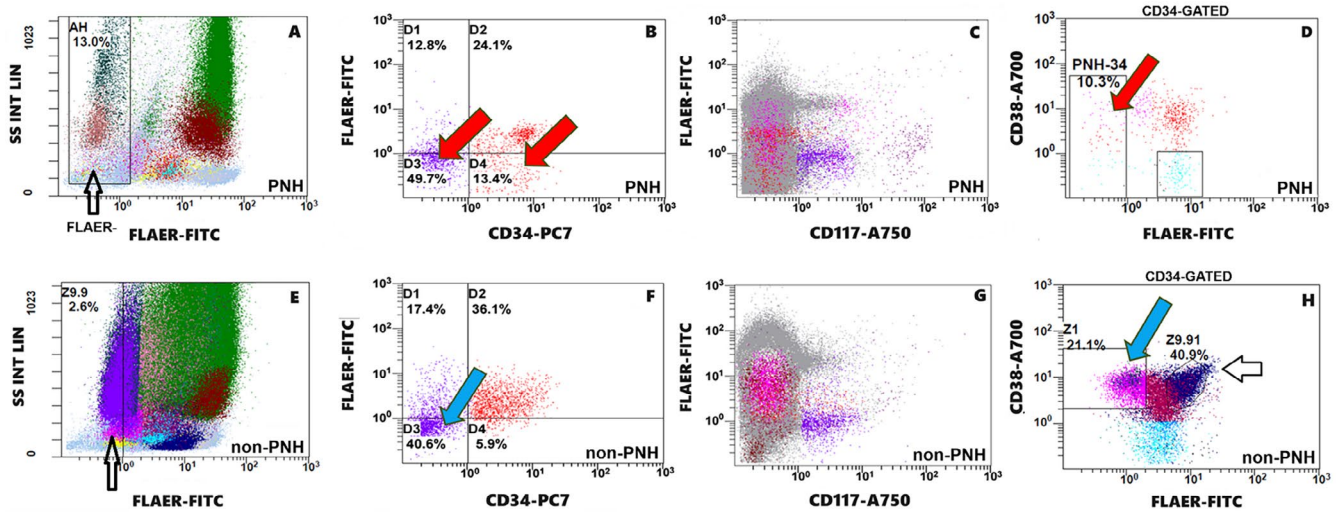

FIGURE 1 | Qualitative comparison of FLAER expression between the BM aspirate of a patient with PNH (A-D) and the BM aspirate of a PNH-negative patient with unexplained neutropenia (reactive, nonmalignant BM aspirate) (E-H). The co-existence of FLAER+ and FLAER-dim or FLAER-negative subpopulations was obvious in the patient with PNH (A, transparent arrow), but in patient with non-PNH only FLAER-dim populations could be observed (E, transparent arrow). In the patient with PNH, CD34+ stem cells showed intermediate FLAER positivity (B, red-colored cells), with lower positivity in CD34-/CD117+/HLA-DR+/CD33+ subpopulation (purple-colored cells) (B, C). The simultaneous presence of FLAER-negative cells was obvious [red arrows, B (gate D4) and D (gate PNH-34)]. CD34++/CD38- cells (light blue–colored cells) were FLAER-positive with decreasing positivity during maturation toward CD34+/CD38+ subpopulation (D, red arrow), but there were also FLAER-negative cells moving toward CD38 positivity (D, gate PNH-34). In PNH-negative patient, a similar pattern of FLAER expression was observed in CD34-/CD117+/HLA-DR+/CD33+ subpopulation (F, G, purple-colored cells), but there was no significant simultaneous detection of a separate cluster with FLAER-negative cells (E-H). Moreover, in PNH-negative patient, CD34++/CD38-/FLAER+ stem cells (light blue–colored cells) seemed to mature in two directions: The one course of maturation leading to CD34+/CD38+ stem cells which start to lose FLAER (H, blue arrow, gate Z1), while the other course of maturation leading toward CD38+/FLAER+ hematogones (H, transparent arrow, gate Z9), which show higher FLAER intensity (these were also CD10+, data not shown) (red-colored: CD34+ stem cells; light blue–colored: CD34++/CD38- stem cells; purple-colored: Immature CD34-/CD117+/HLA-DR+/CD33+ myeloid precursors; green: Mature granulocytes, dark brown: Mature monocytes, pink: CD34+/CD38+ stem cells).

when testing CD34-/CD117+/HLA-DR+/CD33+ subpopulation, a decreased FLAER intensity was revealed (Figure 1B,C, purple-colored cells). Moreover, an increasing FLAER intensity was observed in CD64+/CD14-/HLA-DR+/CD34-/CD117- monocyte precursors (Figure 2C, salmon pink–colored cells), with higher intensity in mature monocytes (Figure 2, dark brown–colored cells) and neutrophils (Figure 3, green-colored cells), but with the consistent simultaneous identification of two discrete subpopulations, one FLAER-positive and one FLAER-negative or dim-positive (Figure 2, light brown–colored PNH monocytes and Figure 3, yellow-colored PNH neutrophils).

3.2 | Non-PNH Patients

In non-PNH patients, both in reactive and pathological BM samples, an association of FLAER intensity and maturation was observed. The side-scatter/FLAER plots identified the existence of CD117-dim cell populations (Figure 1E). When we studied FLAER expression in relation to the maturation stage, CD34++/CD38- and CD34+/CD38+ stem cells showed intermediate FLAER intensity in descending order (more immature CD34+/CD38- stem cells revealed a higher FLAER intensity than more mature CD34+/CD38+ stem cells) (Figure 1H, pink-colored cells, blue arrow). The lowest intensity was detected in CD34-/CD117+/HLA-DR+/CD33+ myeloid precursors (Figure 1F,G, purple-colored cells). Furthermore, an ascending FLAER intensity was observed in CD64+/CD14-/HLA-DR+/CD34-/CD117- monocyte precursors (Figure 2D, blue arrow), with the highest intensity in mature monocytes and neutrophils (Figures 2 and 3, respectively), in the absence of simultaneous FLAER-negative subpopulations. Interestingly, ancestral CD34++/CD38- cells (Figure 1H, light blue–colored cells) had higher FLAER intensity than other myeloid and monocyte precursors (Figure S3). Mast cells, NK cells, and plasma cells were found in the intermediate spectrum (data not shown). In reactive BM aspirates, MDS, and CML, the CD34-/CD117+/HLA-DR+/CD33+ precursors had lower FLAER intensity than AML in diagnosis (AML-Dx) or patients with MDS/MPN, in which a quantitative heterogeneity was observed. Nevertheless, FLAER intensity seemed to follow the same pattern (the highest FLAER attenuation in CD34-/CD117+/HLA-DR+/CD33+ myeloid precursors, intermediate intensity in stem cells and higher intensity in more mature cell subpopulations) (Figure S3). A quantitative assessment of the mean fluorescence intensity (MFI) of FLAER in CD34++/CD38- stem cells, CD34+/CD38+ stem cells, CD34-/CD117+/HLA-DR+/CD33+ myeloid precursors, and CD64+/CD14-/HLA-DR+ monocyte precursors, in comparison with the respective FLAER MFI in mature neutrophils and monocytes,

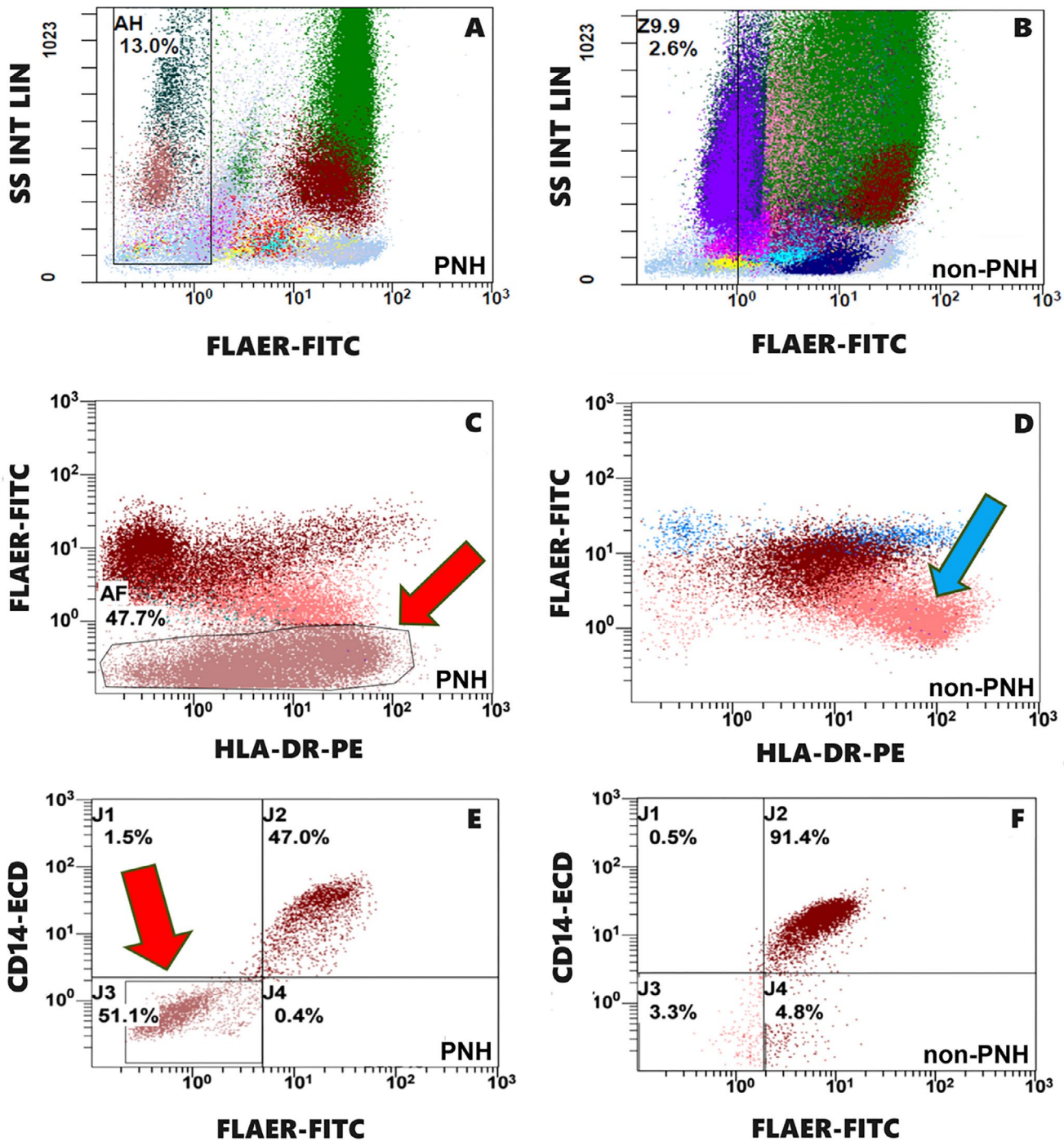

FIGURE 2 | Comparison of FLAER expression in BM mature monocytes and BM monocyte precursors of a patient with PNH (left plots) and a PNH-negative patient with unexplained neutropenia (right plots). The simultaneous presence of FLAER-negative subpopulations was obvious in the patient with PNH (A) compared to the PNH-negative patient (B). An increasing FLAER intensity was observed in CD64+/CD14-/HLA-DR+/CD33+ monocyte precursors (C, D, salmon pink–colored cells) moving toward mature monocytes (dark brown–colored cells) but with the synchronous finding of FLAER-negative cells (C, E, light brown–colored cells) and mature monocytes (dark brown–colored cells) only in the patient with PNH (C, E, red arrow). Plots (C–F) derived from gating of CD34-/CD117-/CD64+/HLA-DR+/CD33+ cells, according to the respective gating strategy (see Figure S2). (dark brown–colored: mature monocytes; salmon pink–colored: CD64+/CD14-/HLA-DR+ monocyte precursors; light brown–colored: PNH-clonal monocytes; green: mature granulocytes, purple-colored: immature CD34-/CD117+/HLA-DR+/CD33+ myeloid precursors).

is depicted in Figure 4. The mean FLAER MFI was displayed for each cell population, according to the diagnostic category (Dx category). In the same figure, the mean FLAER MFI of all subpopulations in patients with PNH was also quoted (referring to the PNH-clonal cells), to highlight the differential FLAER intensity between mature and immature cell populations.

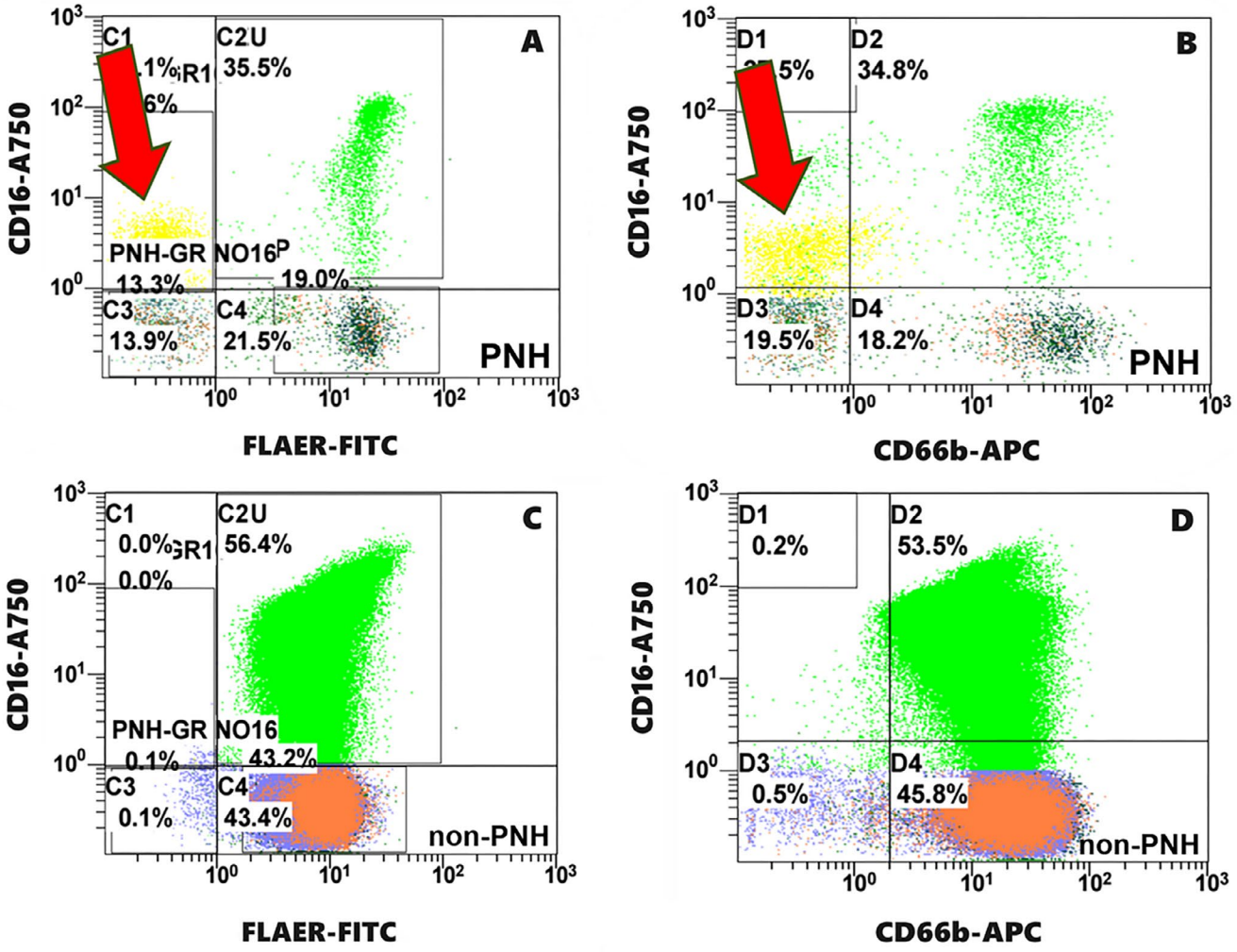

FIGURE 3 | Mature neutrophils in BM aspirates of patients with PNH appeared with a second distinct FLAER-negative/CD16-dim/CD66b-negative cell subpopulation (A, B, yellow-colored cells), compared to the BM neutrophils of reactive BM aspirates which were exclusively FLAER-positive, CD16+, and CD66b+ (C, D, green-colored cells) (yellow-colored: PNH-clonal neutrophils, green-colored: mature neutrophils).

3.3 | Validation of FLAER as Maturation Marker in Granulopoiesis, Analyzing a Case of Acute Promyelocytic Leukemia (APL) at Diagnosis

The maturation pattern of FLAER expression was further confirmed in a patient with APL treated with all-trans retinoic acid (ATRA), where the FLAER-based protocols were applied in serial PB samples over a 20-day period after diagnosis (Figure 5). Abnormal promyelocytes of APL were recognized as CD66b negative and revealed a low FLAER intensity, resembling PNH cells. The administration of ATRA induced not only the upregulation of CD66b, a finding that was initially expected [5], but also the synchronous increase of FLAER intensity during the maturation and differentiation of abnormal promyelocytes (Figure 5).

4 | Discussion

PNH is a rare hematopoietic stem cell disorder resulting from the somatic mutation of the X-linked phosphatidyl-inositol glycan complementation class A (PIG-A) gene [6]. Depending on the severity of the mutation in the PIG-A gene, there is a partial or absolute inability to make glycosylphosphatidyl-inositol (GPI)-anchored proteins including complement-defense structures such as CD55 and CD59 on RBCs and WBCs [6]. Flow cytometric detection of PNH clones has become the gold standard and has played an increasingly important role in the diagnosis, monitoring, and clinical management of patients with PNH [6].

The identification of PNH clones is based on the poor expression of surface proteins, such as CD55 and CD59, and dim or absent binding of FLAER, in mature cell subpopulations [4]. Since the PNH defective red cells undergo hemolysis and because patients often receive red cell transfusions, the clone size is often underestimated in these cells [4]. Contrary to red cells, leukocytes are spared from lysis by complement and unaffected by red cell transfusions, and therefore, are a better reflection of clone size [4]. The PNH clone size in white blood cells (WBCs) is highly variable, and although attempts have been made to determine a "threshold PNH clone size" for a diagnosis of clinical PNH, it should be noted that patients with smaller PNH WBC clones may show signs of clinical PNH, while others with a larger WBC PNH clone size may not [6].

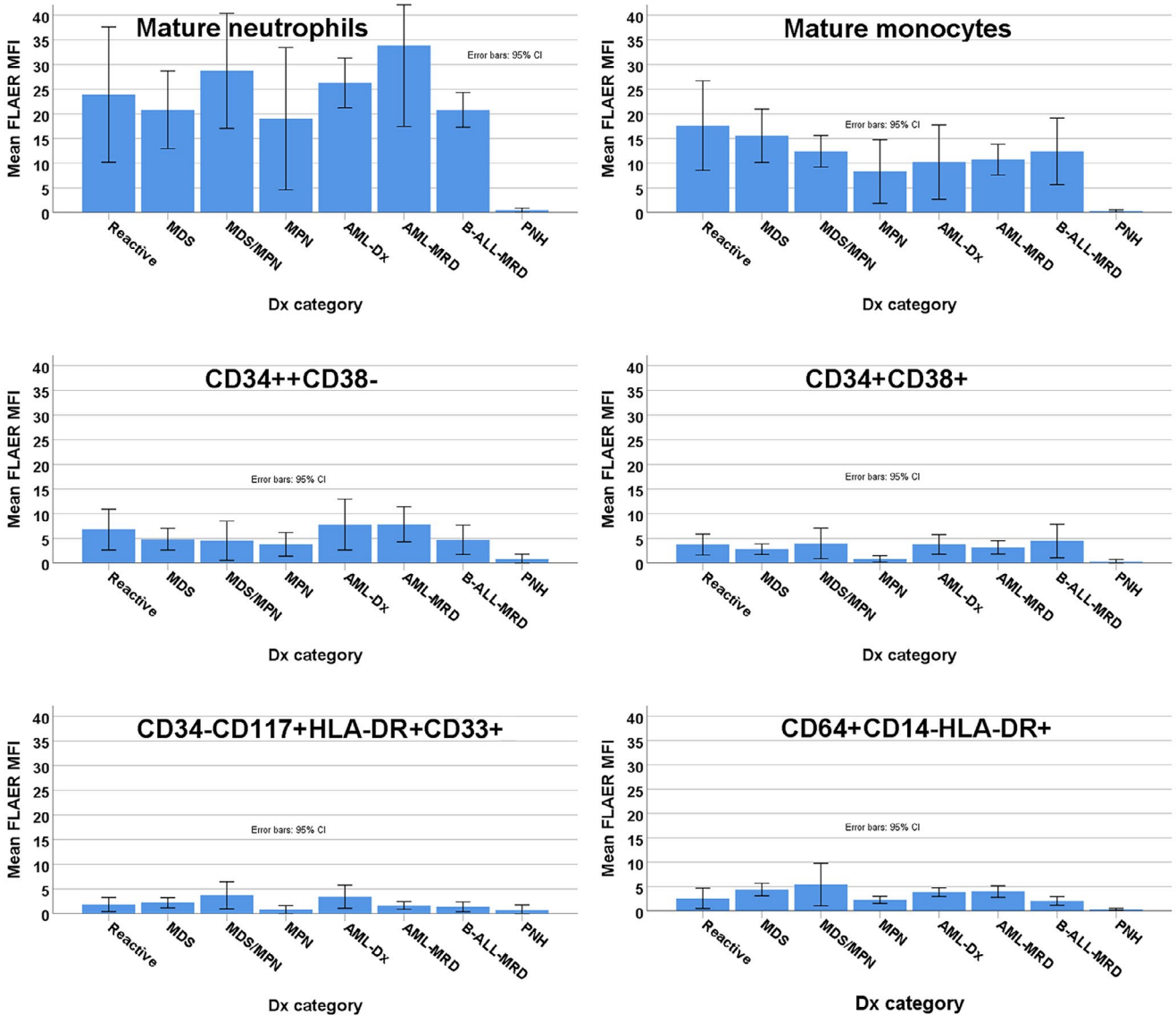

FIGURE 4 | The average mean fluorescence intensity (MFI) of FLAER is shown for CD34++/CD38- stem cells, CD34+/CD38+ stem cells, CD34-/CD117+/HLA-DR+/CD33+ myeloid precursors and CD64+/CD14-/HLA-DR+/CD33+ monocyte precursors, in each different diagnostic category [reactive, MDS, MDS/MPN, MPN (CML cases), AML at diagnosis, AML in MRD assessment, B-ALL in MRD assessment and patients with PNH]. The mean FLAER MFI of mature neutrophils and mature monocytes has also been included, for each diagnostic category, to highlight the difference in FLAER intensity between mature and immature cell populations, and especially CD34-/CD117+/HLA-DR+/CD33+ myeloid precursors, which show the lowest values. The respective FLAER MFI of each population in patients with PNH (referring to the FLAER MFI of PNH-clonal cells) has been included for comparison. Bars represent mean ± SE.

FLAER has been proposed to be the most discriminant marker in PNH, being able to monitor small granulocyte or monocyte PNH clones, especially under conditions such as MDS or bone marrow transplantation (BMT), when traditional GPI-linked surface marker expression can be significantly altered [7]. The ability of FLAER to bind to a wide variety of GPI-linked structures and to be utilized across different leukocyte subsets is remarkable [8]. Even in patients with PNH, the clone size was slightly higher by using FLAER when compared to the non-FLAER-based antibodies panel [2].

There is a limited number of studies on FLAER expression in normal BM aspirates [4, 9, 10]. In PNH evaluation, BM specimens have been generally considered less suitable than blood owing to the variable expression of GPI-linked antigens during hematopoietic cell differentiation. However, it has been shown that FLAER binds to all normal BM white cells, including neutrophil, monocytic, lymphoid, eosinophil, basophil, and plasmacytoid dendritic cell (DC) lineages [4]. Furthermore, when combined with monoclonal antibodies useful in defining different stages of maturation in neutrophils, monocytes, and B cells, it has been suggested that FLAER binding increases with cell maturation in these three cell lineages [4]. In some subpopulations such as early granulocytes, the FLAER binding had been supposed not to be uniform [9]. Our study confirmed these findings and defined the specific pattern of FLAER expression during normal granulopoiesis, which was also consistent in abnormal BM aspirates. CD34+/CD38- stem

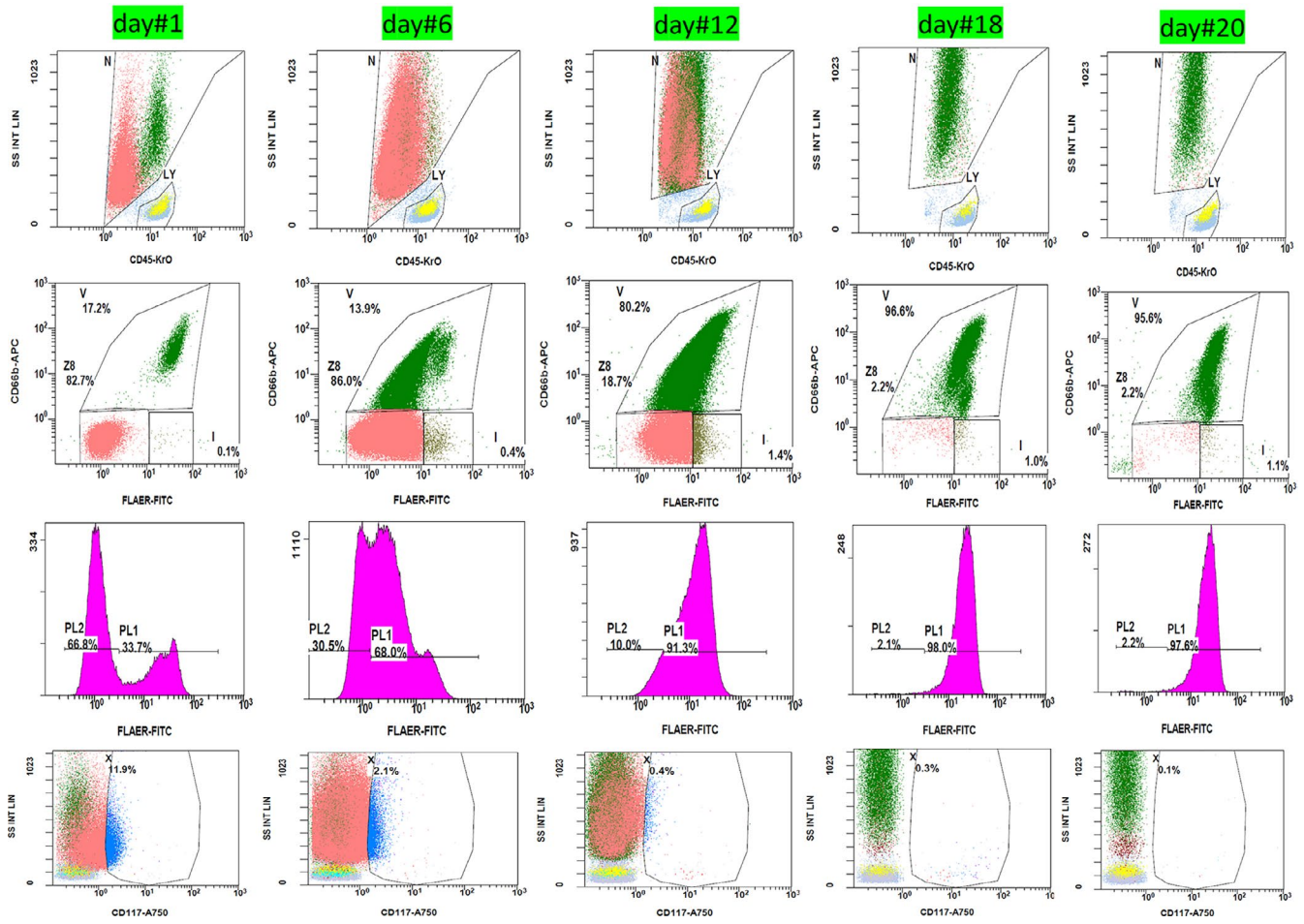

FIGURE 5 | Evaluation of FLAER expression in consecutive PB samples derived from a newly diagnosed patient with APL, who was treated with all-trans retinoic acid (ATRA). The samples were tested randomly, on days #1, #6, #12, #18, and #20 after the initial diagnosis and the administration of ATRA (consecutive columns). Abnormal promyelocytes of APL were initially recognized as CD66b negative and revealed a low FLAER intensity, resembling PNH cells. These were easily discriminated from normal granulocytes (CD66b+/FLAER+). The administration of ATRA induced not only the upregulation of CD66b, a finding that was initially expected but also the synchronous increase of FLAER intensity in the abnormal promyelocytes, which finally maturated and acquired the immunophenotype of normal granulocytes. Also, the consecutive expression of CD117 is depicted in the last row of plots, which shows that the initially dim CD117 expression evolves into negative.

cells were characterized by intermediate FLAER intensity, which reached a nadir in CD117+/CD34- immature myeloid cells. Then, it was gradually increasing, with mature myeloid cells having an increased FLAER positivity. Thus, regardless the fact that we use in our protocols CD14, CD16, and CD66b, which are known GPI-linked surface molecules [9], FLAER seems to be a useful surrogate of GPI-linked markers to correctly identify either immature cell populations or a PNH clone in PNH BM samples.

Although PB remains the preferred source of cells for PNH detection, it has been shown that a careful FC analysis of BM aspirates can provide results for PNH detection and quantitation equivalent to those obtained with PB [4]. Among the limited number of studies on BM aspirates, a limited combination of markers (CD16/CD55/CD45/CD14) has been proposed for the detection of GPI-deficient monocytes and granulocytes in BM specimens submitted for FC evaluation of cytopenias [11]. Dysplastic features in MDS-BM may point to the presence of PNH-type cells, though only a few cases displayed FLAER-negative cells [12]. In the present study, the application of 10-color FLAER-based protocols identified PNH clones through the clear distinction of two subpopulations, one FLAER-positive and one FLAER-dim/negative, in most BM cell populations of patients with PNH. The double discrete subpopulations should constitute a basic criterion for PNH-clone identification, as normal non-PNH FLAER-dim subpopulations can be met. Thus, non-PNH FLAER-dim immature myeloid cells, especially CD117+/CD34- cells, should be carefully interpreted in patients with hematological neoplasms, not to reveal false-positive PNH clones.

As it was presented herein, FLAER expression shows a positive correlation with the granulocytic maturation pattern, with the highest attenuation in CD117+/CD34- immature myeloid precursors. The same was true in our AML cases, a finding that has been partially mentioned in a previous study [13]. In this particular study, CD59 and FLAER expression had been analyzed in 161 patients with AML and CD59 and FLAER deficiencies were identified [13]. Interestingly, compared with nonacute promyelocytic leukemia (non-APL) group, the APL group of patients had more CD59 and FLAER deficiency [13]. That was the reason why we chose to perform consecutive analyses with

the same protocol in the PB of a first diagnosed APL case. An ATRA-driven upregulation of FLAER expression on the abnormal promyelocytes was observed, followed by the concurrent upregulation of CD66b, as it has been previously described [5]. Thus, our findings indicated the potential utility of FLAER as a myeloid maturation and differentiation marker.

In conclusion, the integration of FLAER in BM-associated FC protocols could be helpful in the assessment of hierarchical hematopoiesis. The investigation of FLAER expression in reactive and pathological BM aspirates revealed the existence of normally expected non-PNH FLAER-dim immature myeloid precursors (CD34-/CD117+/HLA-DR+/CD33+), which should be carefully interpreted in hematological disorders. Thus, FLAER could operate as an adjuvant marker in the assessment of hierarchical hematopoiesis, as its expression seems to be associated with the maturation and differentiation of myeloid cells.

Author Contributions

All authors meet the required criteria for authorship.

Ethics Statement

The authors have nothing to report.

Consent

An informed consent was obtained from all patients recruited in the study.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.