Mediterranean Journal of Hematology and Infectious Diseases

Scientific Letters

CD56^{+}/CD38^{+} Neutrophils: Rapid and Specific Flow Cytometric Signature for Chronic Myeloid Leukemia

Keywords: Chronic Myeloid Leukemia (CML); Multiparameter flow cytometry (MFC); CD38; CD56; Neutrophils.

Published: May 01, 2026 **Received: March 31, 2026** **Accepted: April 28, 2026**

Citation: Laganà A., Breccia M., Elia L., Scalzulli E., Bisegna M.L., Ielo C., Buffolino S., Di Lascio A., Germano C.A., Intoppa S., Milani M.L., Martelli M., Breccia M., De Propris M.S. CD56^{+}/CD38^{+} neutrophils: rapid and specific flow cytometric signature for chronic myeloid Leukemia. Mediterr J Hematol Infect Dis 2026, 18(1): e2026049, DOI: http://dx.doi.org/10.4084/MJHID.2026.049

To the editor.

A diagnosis of chronic myeloid leukemia (CML) is usually established in chronic phase, which is characterized by an elevated white blood cell (WBC) count in the peripheral blood (PB). These WBCs are predominantly mature neutrophils, along with their precursors ranging from promyelocytes to neutrophils with band-shaped nuclei.[1-2] CML has traditionally been considered a disease in which flow cytometry provides limited value for differential diagnosis. However, some studies have shown that mature neutrophils derived from the CML stem cells, while expressing typical markers of normal neutrophils (such as CD13, CD33, CD11b, and CD38), also aberrantly express additional markers, including CD56.[3] Moreover, CML leukemic stem cells (LSCs) have been shown to consistently express CD26.[4-5] Recently, Huang *et al.* reported an increased proportion of neutrophils expressing CD38 and bright CD56 (CD56^{bri}CD38^{+}) in bone marrow (BM) samples of CML patients. They found that the CD56^{bri}CD38^{+} neutrophil subset, exceeding 2.0% of total neutrophils, may serve as a highly sensitive and specific flow cytometric marker for identifying CML cases.[6] Hence, in this study, we performed a comprehensive analysis of multiparametric flow cytometry (MFC) data from suspected CML cases, focusing on the expression patterns of CD56 and CD38 on PB neutrophils. This approach aimed to validate the findings of Huang *et al.* and to explore whether evaluating CD56^{bri}CD38^{+} neutrophil subsets in PB could serve as a simple, less invasive flow cytometric signature for early CML detection.

PB cells were stained using a combination of monoclonal antibodies that included CD13/CD33/CD11b/CD15/CD10/CD16/CD56/CD38/ CD34/CD26/CD45/CD117/HLA-DR. Data on standardized 12 color staining combinations were acquired on BD FACS Lyric flow cytometer (Becton Dickinson) and analyzed using the PAINT-A-GATE and FACSDIVA software (Becton Dickinson). A comprehensive and sequential MFC gating strategy was employed to ensure accurate neutrophil identification. Neutrophils were broadly identified by their characteristic CD45/SSC-A distribution, and this population was further refined by verifying the co-expression of CD33 and the presence of mature granulocytic markers, specifically CD10 and CD16.[6-7] The "neutrophil-gated" population refers to this subset of events identified as mature neutrophils based on their CD45/CD33/CD10/CD16 expression and side-scatter properties, while excluding other leukocyte subsets. The percentage of CD56^{bri}CD38^{+} neutrophils was calculated within the gated neutrophil population to allow comparison with previously published approaches,[6] as well as relative to total CD45^{+} WBCs, to assess reproducibility using an easier approach. To address potential inconsistencies and coefficient variation, a high number of events (median 50,000 CD45^{+} cells) was acquired for each sample. Furthermore, to mitigate inter-operator variability, MFC data were independently reviewed and validated by two expert flow cytometrists. Further flow cytometry methods are detailed in the supplementary data. Molecular detection of the BCR::ABL1 transcript was performed by PCR amplification following the primer and protocol recommendations of the BIOMED-1 Concerted Action, with the resulting amplification bands visualized by agarose gel electrophoresis.[8]

This single-center study analyzed MFC data from 41 samples. Among them, 31 were consecutively collected from patients presenting neutrophilic leukocytosis with or without circulating intermediate granulocytic precursors; 17 were later confirmed as newly diagnosed CML cases, while 14 were diagnosed with other hematological disorders (non-CML group). The non-CML cases included patients later diagnosed with polycythemia vera (n=2, 14.3%), atypical CML (MDS/MPN with neutrophilia) (n=2, 14.3%),

Table 1. Clinical characteristics of patients and healthy donors at the time of sample collection. Absolute cell counts correspond to values obtained from automated full blood count (FBC) analyses.

Characteristics	Total Cohort N=41	CML n = 17	Non-CML n = 14	HD-Co n = 10	P-Value
Median Age (IQR) (years)	62 (44.5-74.0)	62.0 (45.0-71.5)	73.0 (63.0-78.3)	31.0 (24.5-54.3)	**< 0.001**
Male (%) Female (%)	23 (56.1) 18 (43.9)	9 (52.9) 8 (47.1)	8 (57.1) 6 (42.9)	6 (60.0) 4 (40.0)	0.94
Median Hb (g/dl) (IQR)	13.1 (10.5-15.5)	12.4 (9.7-13.8)	13.6 (9.9-15.6)	15.0 (13.3-16.7)	**0.031**
Median WBC (x10^9/L) (IQR)	22.8 (10.2-50.2)	50.7 (32.6-153.4)	21.1 (12.8-31.8)	6.3 (4.7-9.2)	**< 0.001**
Median ANC (x10^9/L) (IQR)	14.5 (6.7-41.4)	41.4 (23.4-108.9)	14.1 (7.9-23.1)	3.6 (2.6-6.2)	**< 0.001**
Median ALC (x10^9/L) (IQR)	3.0 (1.7-3.9)	3.6 (3.1-6.6)	1.8 (1.4-3.4)	2.1 (1.7-2.4)	**0.003**
Median AMC (x10^9/L) (IQR)	0.9 (0.5-2.6)	1.9 (1.0-3.5)	0.7 (0.5-1.4)	0.4 (0.2-0.6)	**0.001**
Median AEC (x10^9/L) (IQR)	0.3 (0.1-1.0)	0.7 (0.5-1.9)	0.2 (0.1-0.9)	0.2 (0.1-0.2)	**0.001**
Median ABC (x10^9/L) (IQR)	0.3 (0.1-1.3)	1.5 (0.8-6.0)	0.2 (0.1-0.5)	0.0 (0.0-0.1)	**< 0.001**
Median LUC (x10^9/L) (IQR)	0.4 (0.2-0.9)	0.7 (0.3-1.3)	0.3 (0.2-1.4)	0.1 (0.1-0.2)	**0.007**
Median PLTs (x10^9/L) (IQR)	244.0 (178.0-341.0)	272.0 (196.3-385.0)	310.0 (78.5-397.5)	218.0 (187.3-246.3)	0.46
Median Myeloid Blast-Cells in PB (%)(IQR)	1.0 (0.9-2.0)	1.0 (0.7-1.9)	2.0 (1.0-2.5)	-	0.13
BCR::ABL1 Transcript P210 (%) P190 (%)	 16 (39.0) 1 (2.4)	 16 (94.1) 1 (5.9)	 -	 -	 -
CD56^{bri}CD38$^+$ Neutrophils / Total WBCs (%) (IQR)	0.3 (0.1-2.1)	3.5 (0.4-8.5)	0.2 (0.1-07)	0.1 (0.0-0.3)	**< 0.001**
CD56^{bri}CD38$^+$ Neutrophils / Total WBCs ≥ 2% (%)	12 (29.3)	12 (70.6)	0 (0.0)	0 (0.0)	**< 0.001**
CD56^{bri}CD38$^+$ Neutrophils / Total Neutrophils (%)(IQR)	0.5 (0.2-2.9)	4.0 (0.6-11.5)	0.4 (0.2-0.5)	0.2 (0.1-0.5)	**0.001**
CD56^{bri}CD38$^+$ Neutrophils / Total Neutrophils ≥ 2% (%)	12 (29.3)	12 (70.6)	0 (0.0)	0 (0.0)	**< 0.001**
CD56^{bri}CD38$^+$ Neutrophils (x10^9/L) (IQR)	0.8 (0.1-0.9)	1.1 (0.1-13.6)	0.1 (0.0-0.2)	0.0 (0.0-0.0)	**< 0.001**

Hb, hemoglobin; WBC, white blood cells; ANC, absolute neutrophil count; ALC, absolute lymphocyte count; AMC, absolute monocyte count; AEC, absolute eosinophil count; ABC, absolute basophil count; LUC, large unstained cells; PLTs, platelets; CML, chronic myeloid leukemia; Non-CML, non-chronic myeloid leukemia cases; HD-Co, healthy donors cohort; PB, peripheral blood; IQR, interquartile range.

myelofibrosis (n=1, 7.1%), acute myeloid leukemia (n=1, 7.1%), myelodysplastic syndrome (n=1, 7.1%), chronic myelomonocytic leukemia (n=1, 7.1%), and reactive leukocytosis (n=6, 42.9%). An additional 10 samples from healthy donors served as controls (HD-Co).

Patient and donor characteristics are summarized in **Table 1**. The median age was 62.0 years (IQR, 44.5–74.0), and 23 patients (56.1%) were male. As expected, CML patients exhibited a significantly higher median absolute WBC count at the time of sampling (p<0.001), along with higher median absolute neutrophil count (p<0.001), eosinophil count (p=0.001), and basophil count (p=0.001).

The subset of neutrophil-gated CD56^{bri}CD38$^+$ neutrophils was markedly elevated in CML patients compared to both the non-CML group and healthy donors (HD-Co), in which it was present at minimal levels (p<0.001) (**Table 1**). Within the PB neutrophil compartment, the median percentage of CD56^{bri}CD38$^+$ neutrophils was 4.0% (IQR, 0.6–11.5%) in CML cases, 0.4% (IQR, 0.2–0.5%) in the non-CML group, and 0.2% (IQR, 0.1–0.5%) in the HD-Co (p=0.001) (**Figure 1A**). This difference remained significant even when CD56^{bri}CD38$^+$ neutrophils were expressed as a percentage of total WBCs (rather than within the

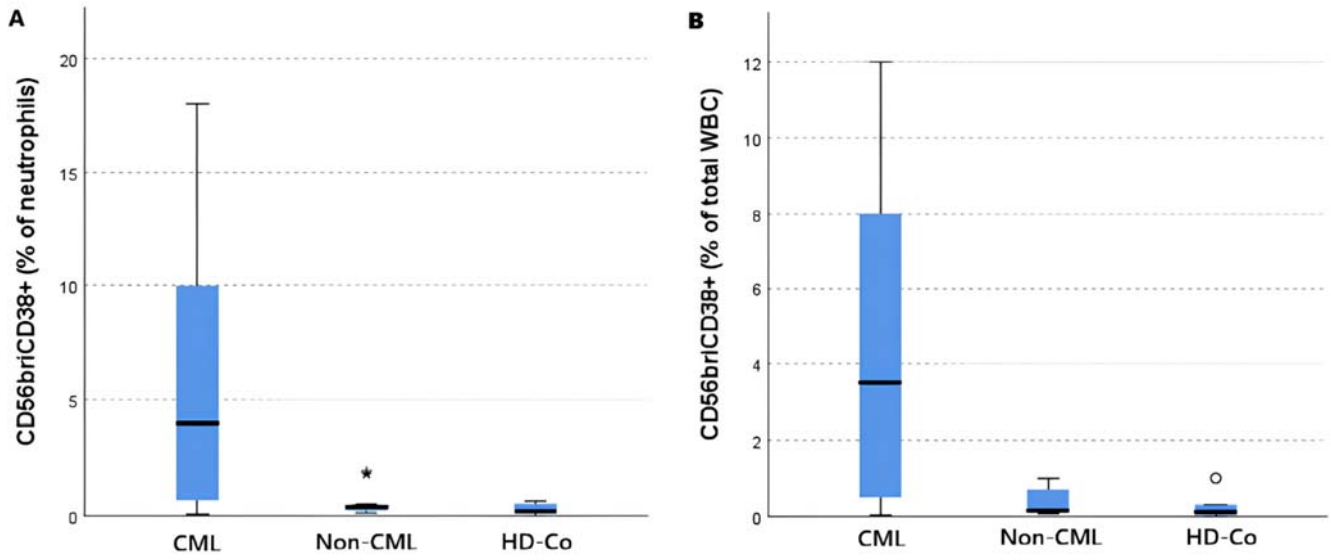

Figure 1. Boxplots of CD56^{bri}CD38^{+} neutrophils in CML, non-CML hematologic disorders, and healthy donor controls (HD-Co)**. A)** Percentage of CD56^{bri}CD38^{+} neutrophils among neutrophils in peripheral blood. **B)** Percentage of CD56^{bri}CD38^{+} neutrophils among total CD45^{+} white blood cells (WBC) in peripheral blood. The asterisk (*) and the open circle (o) represent outliers. Patients with CML exhibited markedly higher frequencies of CD56^{bri}CD38^{+} neutrophils in peripheral blood than both non-CML hematologic disease controls and healthy donors, in both neutrophil-gated and total WBC analyses.

neutrophil gate) (p<0.001) (**Figure 1B**) or as an absolute CD56^{bri}CD38^{+} neutrophil count (p<0.001) (**Table 1**). In fact, as expected, a strong positive linear correlation was observed between the proportion of CD56^{bri}CD38^{+} neutrophils calculated relative to total CD45^{+} positive cells and that relative to the selected neutrophil population (R^2=0.942) (**Supplementary Figure S1**). Receiver operating characteristic (ROC) analysis was performed to evaluate the diagnostic performance of CD56^{bri}CD38^{+} neutrophils for CML prediction. The area under the ROC curve (AUC) for the percentage of CD56^{bri}CD38^{+} neutrophils within the neutrophil gate was 0.842 (95% CI, 0.693–0.991) (p<0.001) (**Supplementary Figure S2A**). A similar AUC value of 0.851 (95% CI, 0.712–0.992) (p<0.001) was obtained when CD56^{bri}CD38^{+} neutrophils were expressed as a percentage of total WBCs (**Supplementary Figure S2B**). The optimal cut-off value providing the best balance between sensitivity and specificity for CML diagnosis was 2.0% for both parameters. At this threshold, specificity and sensitivity were 100.0% (95% CI, 86.2% – 100.0%) and 70.6% (95% CI, 46.9% – 86.7%), respectively, in both cases. Even after adjusting for total white blood cell count, the percentage of CD56^{bri}CD38^{+} neutrophils remained a significant independent predictor of CML, whether expressed relative to the neutrophil population or to total WBCs. Specifically, the percentage of CD56^{bri}CD38^{+} neutrophil was associated with an odds ratio (OR) of 2.92 (95% CI, 1.06–8.03) (p=0.038) when expressed as a proportion of neutrophils, and a OR of 4.80 (95% CI, 1.10–20.97) (p=0.037) when expressed as a proportion of total WBCs.

Notably, in all CML cases, the CD34^{+}/CD38^{-} stem cells in PB, although present at low frequency [median 1.0% (IQR, 0.7-1.9)], expressed CD26, whereas stem cells from the non-CML group lacked CD26 expression.

CD56 is a membrane glycoprotein involved in homotypic adhesion, while CD38 is a cell surface glycoprotein with enzymatic activity in the synthesis and degradation of cyclic ADP-ribose. Aberrant expression of CD56 and co-expression of CD56 and CD38 have been reported in various hematological malignancies, such as abnormal plasma cells in multiple myeloma.[9] As mentioned above, Huang *et al.* highlighted a distinct increase in CD56^{bri}CD38^{+} neutrophils specifically in CML patients, a feature rarely observed in other myeloproliferative neoplasms.[6] We confirmed the findings of Huang *et al.* and demonstrated that this peculiar flow cytometric signature, the presence of CD56^{bri}CD38^{+} neutrophil subset, can also be identified in PB, enabling easier and reliable detection, with the neutrophil-gated 2.0% cutoff that appears applicable to PB specimens as well, promoting an easier approach in clinical practice. Notably, this threshold seems also to be reliably applicable as a percentage of total CD45^{+} WBCs, given the strong positive linear correlation between the proportion of CD56^{bri}CD38^{+} neutrophils calculated relative to total WBCs and that calculated relative to total neutrophils, making its usage more practical in routine analyses. Moreover, we confirmed previous reports that the CD34^{+}/CD38^{-}/CD26^{+} LSCs population in PB represents a hallmark of CML, even when present at low frequency.[4-5]

This flow cytometric approach should be considered as a supportive screening tool to prioritize gold-standard molecular or cytogenetic assays for CML diagnosis. Its clinical utility may lie in providing a rapid diagnostic suspicion, particularly when cytomorphological

evaluation is inconclusive or lacks the classical features of CML, or when molecular results might be immediately unavailable or excessively delayed. In these scenarios, identifying this signature can effectively prioritize testing in patients with unexplained neutrophilic leukocytosis and ambiguous morphological profiles. This study presents several limitations, including the small size of the CML cohort and its single-center nature. Moreover, while the heterogeneity of the non-CML comparator group reflects real-world clinical practice, this cytometric approach requires further validation in larger, multicenter external datasets to fully characterize its diagnostic performance and scalability. Nevertheless, our data successfully validate the findings previously reported by Huang et al.[6] in a different cohort, confirming that the flow cytometric identification of $CD56^{bri}CD38^{+}$ neutrophil subsets in the PB may represent a rapid and cost-effective screening tool to raise suspicion of CML and to prompt timely confirmatory molecular assays for BCR::ABL1 transcript detection and/or cytogenetic testing for t(9;22) identification.

Conflicts of Interest. Massimo Breccia received honoraria from Novartis, Incyte, Pfizer, BMS, AOP, Abbvie, GSK. Maurizio Martelli received honoraria from Roche, Gilead Sciences, Novartis, Abbvie, Incyte, BeiGene, Takeda, and Bristol Myers Squibb/Celgene. The other authors declare no conflict of interest.

Author Contributions. Alessandro Laganà and Matteo Breccia: Writing the original draft, data collection and interpretation, and formal analysis. Loredana Elia: Molecular data collection, interpretation, and contribution to lab work. Emilia Scalzulli and Maria Laura Bisegna: Followed the patients. Claudia Ielo: Molecular data collection and contribution to lab work. Sonia Buffolino and Attilio Di Lascio: Molecular data analysis. Concetta Anna Germano, Stefania Intoppa, and Maria Laura Milani: Flow cytometry data collection, acquisition, and interpretation. Maurizio Martelli and Massimo Breccia: Supervision and manuscript editing. Maria Stefania De Propris: Data collection and interpretation, conceptualization, investigation, and writing the original draft.

Data Availability Statement. The data that support the findings of this study are available in the text and from the corresponding author, Massimo Breccia, upon reasonable request.

Informed Consent. Written informed consent was collected according to local practice

Acknowledgments. Concetta Anna Germano, Stefania Intoppa, Attilio Di Lascio and Maria Laura Milani were supported by ROMAIL ONLUS

Ethics Approval Statement. This study was conducted in accordance with the principles outlined in the Declaration of Helsinki. As a non-interventional study using data collected as part of routine diagnostic procedures, in accordance with local regulations, it did not require ethics committee approval.

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Competing interests: The authors declare no competing interest.

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