NEUTROPHIL SCATTERING DATA DRIVEN PRE-MICROSCOPIC FLAGGING OF ACUTE LEUKEMIC CASES

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ABSTRACT

Background: The hematology analyzer, Sysmex XN-1000, generates white blood cell count with varying scattering intensities during a complete blood count (CBC) analysis. **Objectives:** The objectives of the study were to study the predictive role of median and coefficient of variation of neutrophil scattering items in blood samples for differentiation of leukemic subjects. **Methods:** We evaluated six neutrophil scattering parameters: neutrophil side scatter mean intensity, neutrophil side fluorescence light (SFL) mean intensity, neutrophil forward scatter mean intensity, neutrophil side scatter area distribution width (NE-WX), neutrophil SFL area distribution width (NE-WY), and neutrophil forward scatter area distribution width (NE-WZ), measured in white blood cell differential scattergram generated by the hematology analyzer (Sysmex XN-1000) at an academic medical center. **Results:** We collected 433 blood samples from acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) cases and normal controls. AML group showed highly significant differences in the mean values compared with the control group. Out of six neutrophil scattering items, NE-WX, NE-WY, and NE-WZ showed high efficiency, with area under the curve (AUC) values of 0.764, 0.748, and 0.757, respectively, to differentiate AML from ALL cases and control groups. When comparing combined acute leukemia cases (AML plus ALL) with the control group, NE-WX, NE-WY, and NE-WZ generated highly significant AUC values (0.840, 0.884, and 0.801, respectively). **Conclusion:** The neutrophil scattering parameters generated during CBC analysis provide a new tool for the prediction of acute leukemia and its lineage. (REV INVEST CLIN. 2020;72(1):37-45)

Key words: Acute leukemia. Sysmex. Hematology analyzer. Neutrophil scattering items.

INTRODUCTION

Acute leukemias represent a heterogeneous group of blood cancers with uncontrolled proliferation of immature and/or abnormal hematopoietic cells

without any relationship with a specific age group, although there is a predominance of myeloid neoplasms in adults while lymphoblastic malignancies are more common in children. To classify acute leukemia, a complex approach is followed, including

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various laboratory techniques: peripheral blood and bone marrow morphological examination, immunophenotyping by flow cytometry and/or immunohistochemistry, and identification of specific molecular and cytogenetic abnormalities. Cell morphology is usually the first and one of the most important diagnostic parameters generated by the hematology laboratory. Nevertheless, over the past decades, due to increasing economic pressures and workload and with the progress in automated hematology analyzers for blood cell counting and differential, the practice of peripheral morphologic examination steadily decreased. Moreover, cytochemistry and later flow cytometry largely replaced morphologic examination. The presence or absence of abnormal cells requires a trained hematologist/pathologist to examine meticulously the slide under a microscope and reports the findings. This generally takes 2-4 h or more before the clinician obtains information about a suspected case of acute leukemia. When patients with acute leukemia present with very high white blood cell (WBC) count, it is easier to suspect it; however, when the WBC is normal or low, the index of suspicion for the presence of blasts is rather low and examination of peripheral blood count is usually not employed, thereby delaying the diagnosis. Along the above-said high counts and abnormalities, abnormal maturation of neutrophils on both ends (nuclear [such as abnormal nuclear segmentations, Auer rods], and cytoplasmic side [such as a defect in primary and/or secondary granule's constituents]) is also noted in acute leukemia, especially in acute myeloid leukemia (AML), which strengthens the suspicion of acute leukemia¹⁻³.

The modern hematology analyzers flag the abnormal complete blood count (CBC) analysis and prompt the technologist to prepare the peripheral blood film for validation of the flagged finding. Sysmex XN-series CBC analyzers are flow cytometry-based instruments, in which the blood sample is mixed with special reagents (surfactants) that cause the hemolysis of red blood cells (RBC) and platelets and facilitate the penetration of the reagent (into the WBCs) used in the following step. Then, a specific reagent containing a fluorescence dye (Polymethine dye) is used which stains the cell's nucleic acid content either in the nucleus or organelles such as mitochondria and rough endoplasmic reticulum⁴. A laser light of 633nm is used to hit the cells flowing in

a line by a stream of fluid and sequentially irradiated by a laser. Flowing of cells in light causes their scattering at various angles and also the emission of fluorescence light from the fluorescent dye, both of which are measured and processed for the generation of a 2D scatterplot on the main screen of the analyzer (Fig. 1). In addition, the values for these measurements can be viewed in background data under different titles as WBC or white blood cell differential (WDF) channel research parameters. The value of forward scattering light (FSC) and side-scattering light (SSC) are proportional to the size and internal complexity of the cell, respectively. In addition, the intensity of side fluorescence light (SFL) reflects the cell's DNA/RNA content⁵.

In this study, to focus the abnormal maturation of neutrophils in a quantitative manner for the prediction of acute leukemia, we selected the values (parameters) of FSC, SSC, and SFL for neutrophils: neutrophils complexity (neutrophil side scatter mean intensity [NE-SSC]) and the width of dispersion (area) of neutrophils complexity (neutrophil side scatter area distribution width [NE-WX]), neutrophils fluorescence intensity (neutrophil side fluorescence light mean intensity [NE-SFL]) and width of dispersion (area) of neutrophils fluorescence (neutrophil side fluorescence light area distribution width [NE-WY]), and neutrophils size (neutrophil forward scatter mean intensity [NE-FSC]) and width of dispersion (area) of neutrophils size (neutrophil forward scatter area distribution width [NE-WZ]). The value of NE-SSC, NE-SFL, NE-FSC, NE-WX, NE-WY, and NE-WZ varies in healthy and ill population and can be investigated as an important predictive marker in different hematological malignancies. There are some emerging data on the utilization of these non-reportable research parameters in sepsis⁶⁻¹⁰, myelodysplastic syndrome¹¹⁻¹⁵, and severe infections¹⁶⁻¹⁹. Studies concluded that such biomarkers might provide useful information for the early diagnosis and adequate management of these disorders. These parameters have been used in acute and chronic leukemia, but they have not been extensively studied especially in big data sets. The ability of Sysmex XN-1000 to generate WBC scattering parameters during a routine CBC analysis can be used to identify leukemic samples and thus minimize the delay in the diagnosis of such cases. The objective of this study was to evaluate the potential of NE-SSC, NE-SFL, NE-FSC,

Figure 1. Position of different cell populations on white blood cell differential scatterplot by Sysmex XN-1000 hematology analyzer, represented by oblong colored areas. X-axis shows side scatter (SSC) of laser light, Y-axis represents side fluorescence (SFL) and forward scatter (FSC). Through different colors, clusters of various types of leukocytes are represented: light blue for neutrophils, pink indicates lymphocytes, monocytes by green, and red for eosinophils. (A) Neutrophil scattering parameters are shown by arrow for granularity (SSC) on X-axis: NE-X and neutrophil side scatter area distribution width, while pulse neutrophil fluorescence light intensity (SFL) on Y-axis: NE-Y and neutrophil side scatter area distribution width. (B) Scattering items for size of neutrophils are presented with arrow on X-axis: NE-Z and neutrophil forward scatter area distribution width.

NE-WX, NE-WY, and NE-WZ to predict acute leukemia blood samples.

METHODS

Subjects and procedures

Study subjects were individuals suspected of having acute leukemia presenting to the hematology outpatient clinic of an academic research center (NIBD and BMT) in Karachi, Pakistan, for 18 months. Healthy blood donors were included as control subjects. The Institutional Ethics Committee approved the study and informed consent was obtained from study subjects. Clinical and demographic information of patients and controls was recorded.

Blood samples were collected in K₃EDTA plastic tubes (Becton Dickinson, USA) and analyzed on XN-1000 (Sysmex Co., Kobe, Japan) within an hour of sample

collection. The final diagnosis of acute myeloid (AML) and acute lymphoblastic (acute lymphocytic leukemia [ALL]) leukemia was made based on peripheral blood film, bone marrow examination, confirmed through immunophenotyping (by flow cytometry or immunohistochemistry), cytogenetics, and/or molecular genetics. Non-leukemic patients and those with a history of chemotherapy and blood transfusion were excluded from the study. Acute leukemia cases having > 1000 WBC count and at least 5% immature/abnormal WBCs cell count in peripheral blood with complete diagnostic workup were included in this study. Data on standard CBC parameters were recorded. The values of NE-SSC, NE-SFL, NE-FSC, NE-WX, NE-WY, and NE-WZ were collected.

Statistical analysis

SPSS 20.0 was used for the analysis of data and $p < 0.05$ was considered significant. Calculations of mean, standard deviation (SD), and significance

Table 1. Mean, SD, and significance (p-) values for classical CBC items provided by CBC (Sysmex XN-1000) analyzers, separated by study groups

Parameters	Control (Mean $\pm$ SD)	AML (Mean $\pm$ SD)	ALL (Mean $\pm$ SD)	p-value (AML vs. ALL)
Hb	13.91 $\pm$ 1.25	7.58 $\pm$ 2.60*	5.85 $\pm$ 3.46*	< 0.001
RBC ($10^{12}/L$)	4.85 $\pm$ 0.43	3.53 $\pm$ 2.35*	5.29 $\pm$ 3.00*	< 0.001
PCV	42.64 $\pm$ 3.49	25.19 $\pm$ 7.61*	24.73 $\pm$ 7.63*	0.057
MCV	88.04 $\pm$ 3.27	89.92 $\pm$ 10.06*	83.93 $\pm$ 9.12*	0.388
MCH	28.71 $\pm$ 1.09	29.32 $\pm$ 3.43*	27.35 $\pm$ 2.91*	0.072
MCHC	32.63 $\pm$ 1.19	32.47 $\pm$ 2.02*	32.63 $\pm$ 1.92*	0.766
WBC ($10^9/L$)	7.63 $\pm$ 1.25	37.15 $\pm$ 63.58*	70.91 $\pm$ 107.47*	< 0.001
PLT ($10^3/\mu L$)	278.70 $\pm$ 53.60	59.48 $\pm$ 83.57*	53.74 $\pm$ 62.92*	0.684
NEUT# ($10^3/\mu L$)	4.35 $\pm$ 0.93	9.73 $\pm$ 27.80	3.24 $\pm$ 4.22*	0.350
LYMPH# ($10^3/\mu L$)	2.45 $\pm$ 0.54	8.24 $\pm$ 12.67*	47.75 $\pm$ 77.40*	< 0.001
MONO# ($10^3/\mu L$)	0.53 $\pm$ 0.11	19.51 $\pm$ 40.24*	20.08 $\pm$ 42.25*	0.031
EO# ($10^3/\mu L$)	0.23 $\pm$ 0.14	0.16 $\pm$ 0.89*	0.13 $\pm$ 0.28*	0.581
BASO# ($10^3/\mu L$)	0.04 $\pm$ 0.01	0.71 $\pm$ 0.19*	0.15 $\pm$ 0.38*	< 0.001
NEUT% (%)	56.92 $\pm$ 6.03	24.80 $\pm$ 20.59*	13.11 $\pm$ 17.23*	< 0.001
LYMPH% (%)	32.28 $\pm$ 5.72	37.89 $\pm$ 23.12	64.35 $\pm$ 22.06*	< 0.001
MONO% (%)	7.09 $\pm$ 1.15	36.45 $\pm$ 23.83*	21.06 $\pm$ 18.03*	< 0.001
EO% (%)	3.14 $\pm$ 1.78	0.67 $\pm$ 1.97*	0.49 $\pm$ 0.95*	< 0.001
BASO% (%)	0.55 $\pm$ 0.25	0.17 $\pm$ 0.32*	0.22 $\pm$ 0.29*	0.007
IG# ($10^3/\mu L$)	0.26 $\pm$ 0.01	1.79 $\pm$ 4.63*	0.73 $\pm$ 1.50*	< 0.001
IG% (%)	0.34 $\pm$ 0.19	4.52 $\pm$ 6.61*	1.76 $\pm$ 2.93*	< 0.001

*p-value < 0.05 against control. CBC: complete blood count; SD: standard deviation; ALL: Acute Lymphocytic Leukemia; AML: Acute Myeloid Leukemia; RBC: red blood cell; IG: immature granulocyte.

(p-) values against control and between acute leukemia groups were carried out using SPSS. To determine the predictive ability of neutrophil scattering items for acute leukemia, receiver operating characteristic (ROC) curve and the area under the curve (AUC) were generated.

RESULTS

A total of 433 subjects were analyzed. Of these, 76 were diagnosed as ALL, 82 as AML, and 275 were healthy subjects (control group). The control-to-patient group (ALL and AML) ratio was 1.76. The mean ages of patients and controls were 32.76 ± 19.9 years and 29 ± 11.5 , respectively. The male-to-female ratios in patients and control group were 1.2 and 1.3, respectively. Values of mean, SD, and significance (p-value)

for classical CBC parameters among study groups are presented in table 1, and those for neutrophil scattering items generated by the Sysmex XN-1000 hematology analyzer are presented in table 2. Significance (p-value) was calculated and presented in two separate manners: (1) for each disease group (AML or ALL) compared with the control group and symbolized by “*” if found significant; and (2) between disease groups (AML vs. ALL), which is shown in the last column of tables 1 and 2.

Differentiation between disease group (AML or ALL) and healthy control group

A significant increase was observed in several classical (routine) and neutrophil scattering CBC items in both AML and ALL compared to healthy controls

Table 2. Mean, SD, and significance (p-) values for six neutrophil scattering parameters provided by CBC (Sysmex XN-1000) analyzers, separated by study groups

Parameters	Control (Mean $\pm$ SD)	AML (Mean $\pm$ SD)	ALL (Mean $\pm$ SD)	p-value (AML vs. ALL)
[NE-SSC(ch)]	149.17 $\pm$ 5.55	141.24 $\pm$ 13.50*	149.64 $\pm$ 9.25*	< 0.001
[NE-SFL(ch)]	46.98 $\pm$ 2.21	54.32 $\pm$ 19.33*	50.71 $\pm$ 8.30*	0.033
[NE-FSC(ch)]	86.95 $\pm$ 3.91	72.35 $\pm$ 11.25*	80.89 $\pm$ 7.03*	0.505
[NE-WX]	304.64 $\pm$ 17.13	432.54 $\pm$ 125.52*	386.73 $\pm$ 108.57*	0.699
[NE-WY]	609.48 $\pm$ 26.96	1364.63 $\pm$ 769.58*	1226.47 $\pm$ 616.40*	0.881
[NE-WZ]	610.18 $\pm$ 42.60	820.95 $\pm$ 249.46*	721.08 $\pm$ 203.64*	< 0.001

*p-value < 0.05 against Control. ALL: Acute Lymphocytic Leukemia; AML: Acute Myeloid Leukemia; SD: standard deviation; NE-SFL: neutrophil side fluorescence light mean intensity; NE-FSC: neutrophil forward scatter mean intensity; NE-SSC: neutrophil side scatter mean intensity; NE-WX: neutrophil side scatter area distribution width; NE-WY: neutrophil side fluorescence light area distribution width; NE-WZ: neutrophil forward scatter area distribution width.

(Table 1). Patients had significantly higher rates of leukocytes, lymphocytes, and monocytes in both types of acute leukemia; hemoglobin and platelet counts were decreased while neutrophil count was not significantly modified. A significant difference was also noted between disease and reference (control) groups, whatever the cause, for these neutrophil scattering items (Table 2): NE-SFL, NE-WX, NE-WY, and NE-WZ were increased, while NE-SSC and NE-FSC were slightly decreased or found near normal ranges.

Both AML and ALL disease groups mainly followed the same trend in routine CBC items, except for the lymphocyte percent count, which remained unmodified for AML. Interestingly, four neutrophil scattering items, NE-SFL, NE-WX, NE-WY, and NE-WZ, had higher values, and NE-FSC had lower values (against control for AML) compared to ALL, while exceptionally NE-SSC remained unaffected in ALL patients.

Differentiation between AML and ALL

Considering the whole population of our disease data set, from classical CBC items, the level of hemoglobin and counts for RBCs, WBCs, absolute lymphocyte, absolute basophil, percent neutrophil, lymphocyte, monocyte, and eosinophil, absolute, and percent immature granulocyte (IG) were found to be significantly different when comparing AML to ALL (Table 1). Leukocytes, absolute, and percent lymphocytes were significantly higher in ALL ($p < 0.0001$), while for

AML, percent monocyte, absolute, and percent IG were found with significantly increased counts.

As shown in table 2, Sysmex XN-1000 analyzer provided discriminating values for only NE-SSC and NE-WZ between AML and ALL.

ROC curves

Neutrophil scattering items were used to establish ROC curves (Fig. 2), differentiating between disease group and healthy controls (Fig. 2: AL, AML, and Non-AML [ALL plus control]); AML, and ALL and Non-ALL (AML plus control). In general, three out of six neutrophil scattering parameters (NE-WX, NW-WY, and NE-WZ) provided significant discrimination with higher AUC values. In differentiating acute leukemia (disease) groups from non-disease (control) group, excellent discriminating power as AUC values were achieved for NE-WX, NE-WY, and NE-WZ: 0.840, 0.884 and 0.801, respectively (Fig. 2). For disease groups, less-significant AUC values of 0.601, 0.680, and 0.558 for NE-WX, NE-WY, and NE-WZ, respectively, in ALL cases were found, while for AML, NE-WX, NE-WY, and NE-WZ provided significant AUC values of 0.764, 0.748, and 0.757, respectively.

Increase in width and shifting of neutrophil's scattering area in disease group

The light-scattering intensities are processed and presented on the main screen of the analyzer as

Figure 2. Receiver operating characteristic curves and area under the curve values of neutrophil scattering items, neutrophil side scatter area distribution width, neutrophil side fluorescence light area distribution width, and neutrophil forward scatter area distribution width provided significant results for all three types of discrimination (AL: acute leukemia from control; acute myeloid leukemia [AML] from Non-AML [acute lymphocytic leukemia (ALL) plus control]; ALL from Non-ALL [AML plus control]).

scatterplots. Figure 3 shows the increase in width and/or shifting of neutrophil's scattering area on WDF scatterplots in AML and ALL (by comparing white circles in AML and ALL against the control group). As noted in figure 3, compared to healthy subjects, the increase in width of neutrophil's scattering area was significantly prominent for both of our disease groups, while shifting of neutrophil's scattering area was comparatively less perceptible. Between disease groups, AML showed a highly discriminating scattering trend for neutrophil's area compared to a moderate increase in width of scattering area in patients with ALL.

DISCUSSION

Despite the current need for extensive testing for the complete classification approach of acute leukemias,

physicians need first to know the lineage of the suspected case of leukemia. Therefore, laboratory testing usually starts with morphology and flow cytometry of the patient's blood and/or bone marrow samples before advancing to the molecular and cytogenetic testing. Flow cytometry, however, in itself is not a routine test, and is typically operated during regular work hours and only feasible in limited well-equipped laboratories and hospitals due to the requirements of experienced personnel and modern instrumentation, problems, which are more pronounced in developing countries.

Among complex tests for the diagnostic workup of acute leukemias, hematology analyzers for CBC analysis provide WBC research (scattering) parameters that have the potential to detect morphological changes in these cells. These scattering items of WBCs, especially neutrophils, have been useful in the prediction

Figure 3. White blood cell differential scatterplots for **A** = Healthy subject (without any abnormal or immature white blood cell), **B** = Acute myeloid leukemia, and **C** = Acute lymphocytic leukemia. White circles indicate the neutrophil's scattering area.

A Control

B AML

C ALL

of sepsis and neutrophil dysplasia^{9,11,14,20}. Their usefulness was not much studied for the differentiation of normal samples in comparison with immature

neutrophils, including blast cell populations, in distinguishing various types of leukemic disorders or for their detection against leukemoid reaction and infections.

A study by Yang et al. evaluated white cell scattering items for differentiation of acute leukemia lineage using Coulter D × H800 analyzer. In this study, the authors derived a model based on 21 items and reported very high (100%) specificity and sensitivity for differentiation of acute promyelocytic leukemia (APL) cases, while for ALL, comparably less significant specificity and sensitivity were achieved²¹. Another study by Virk et al. reported the clinical utility of the white cell scattering items, scattergrams, and flags for screening of AML cases with significant specificity²².

The findings from our study are promising for acute leukemia since our neutrophil scattering items were not only able to detect the presence of acute leukemia but also predict its lineage (AML or ALL). Of particular importance was the performance of NE-WX, NE-WY, and NE-WZ, which was found with significantly higher values for AML followed by ALL compared with healthy subjects. Most immature cells in AML and ALL, especially APL cells, have been found to be counted near or in the neutrophil population area, which caused shifting and particularly increase in width of the neutrophil's scattering area (Fig. 3)²³. Consequently, this area in AML cases would be a mixture of AML immature/abnormal cells and neutrophils, thus generating higher values for NE-WX, NE-Y, and NE-WZ.

A number of studies have reported the key importance of WBC morphology in the diagnosis of hematological diseases. While the microscopical review of every CBC differential is not feasible, especially in labs with heavy workload, labs have now built their knowledge and expertise for the use of modern hematology analyzers to assess these morphologic characteristics in a fast, inexpensive, automated, and quantitative manner. In addition to obtaining a straightforward interpretation of results and numerical decision rules of the quantitative analysis, a screening algorithm can be implemented using the criteria of the scattering items based on big data sets. Aiming for early prediction of acute leukemia cases and its sub-classification in AML or ALL, the present study showed a potential clinical utility of these scattering items.

This study has some limitations. First, the effect of sepsis in study cases was not excluded by any reference method. However, as reported in some studies^{9,20},

sepsis caused only a moderate increase in values of neutrophil research items, particularly parameters related with neutrophil's area width (NE-WX, NE-WY, and NE-WZ), compared against discriminatively higher values in patients with acute leukemia (where immature/abnormal WBC counted in neutrophil scattering area produced very high values). Second, any changes in research parameters based on the disease subgroup at the time of diagnosis of acute leukemia could not be evaluated in the present study. The investigation of these research (extended) parameters in differentiation and identification of subgroups of acute leukemia can provide important information, such as the prediction power and detection sensitivity of these research items for subtypes of acute leukemia, and therefore, should be addressed in the future.

In summary, we here propose a new solution for the screening of acute leukemia cases and differentiating their lineage on neutrophil scattering parameters generated along regular CBC-diff. These findings may be of clinical importance in early, cost-effective, and more accurate diagnosis (by avoiding unnecessary tests) of acute leukemia patients, especially in resource-constrained setups.

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