Detection of Apoptotic Lymphocytes Through Sysmex XN-1000 As a Diagnostic Marker for Mononucleosis Syndrome

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Background: The infectious mononucleosis (IM) includes clue elements, apoptotic and atypical lymphocytes. In IM, the evaluation of dot plot provided by Sysmex XN-1000 analyzer revealed a stretched lymphocytic cluster, white cell differential channel (WDF), on cytogram. **Methods:** In this study, we analyzed 698 samples that include 39 IM, 76 chronic lymphoproliferative disorders, 25 nonclonal lymphocytosis, and 40 healthy donors. Five hundred eighteen samples with other diseases or interference were evaluated. The algorithm was validated on 40,000 files that were received from internal database of Sysmex-Dasit. **Results:** The analysis of flow cytometry standard (FCS) files in WDF channel and presumed apoptotic lymphocytes counts on side scatter/forward scatter (SSC/FSC) and SSC/SFL (where SFL is side fluores-

cence) dot plot revealed excellent correlation among apoptotic cells on peripheral blood smear ($R^2 = 0.79$ and 0.75). There was a variation of positional parameters in lymphocyte clusters WX, WY, and WZ. If $WX-SSC > 500$ and $WY-SFL > 1,000$ and $WZ-FSC > 700$, specificity equals to 99% and sensitivity equals to 100%. If nucleated red blood cell (NRBC) $< 0.03 \times 10^3/\mu\text{L}$, specificity equals to 100%. In received files, positives were 1% adding the simultaneous presence of a percentage of events in the two gates relating to the apoptotic lymphocytes of 1.2% of WBC. **Conclusion:** On Sysmex XN-1000, dot-plot observation allowed immediate detection of IM. Meanwhile, an algorithm based on the parameters on these plots can be calculated with excellent performance. *J. Clin. Lab. Anal.* 30:779–793, 2016. © 2016 Wiley Periodicals, Inc.

Key words: Epstein–Barr; cytomegalovirus; toxoplasma; Downey; CD8+; suppressor; Hoagland; polymethine; WDF; WNR

INTRODUCTION

IM is a benign, acute, self-limiting disease that mainly occurs in children and young adults (1). Although it is estimated that 85–90% of the cases of mononucleosis syndrome are caused by the Epstein–Barr virus (EBV) (2,3), it is observed that infections by other agents, such as cytomegalovirus, *Toxoplasma gondii*, adenovirus, rubella, and hepatic viruses, can develop with similar hematological and clinical signs (4).

Presentation among children and youth is represented by the classic triad—fever, pharyngitis, and lymphadenopathy—often accompanied by asthenia with prolonged malaise and, later, splenomegalia and hepatomegalia. Among the adults and elderly, however, there are few symptoms that can be attributed to the oropharynx and few or no adenopathies, while anicteric hepatitis is more frequent.

The classic triad of symptoms, as well as a lymphocytosis $> 50\%$ with atypical lymphocytes $\geq (10)\%$, constitutes useful criteria, developed by Hoagland, for the diagnosis of mononucleosis syndrome (5). However, previous studies have shown a substantial percentage of patients with serological evidence of mononucleosis infection without clinical manifestations or quantitative hematological changes on the peripheral blood smear (5)—even with the presence of the typical morphological anomalies represented—by reactive lymphocytes, classified into three subgroups by Downey (6):

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Fig. 1. Apoptotic lymphocyte in a patient with mononucleosis syndrome. Peripheral blood smear—May-Grünwald-Giemsa stain 1,000 $\times$.

- *Downey Type I*: plasmacytoid appearance, only slightly larger than small lymphocytes, with round or oval nuclei and chromatin clumped into small masses and basophilic cytoplasm, often frothy due to the presence of fine vacuoles.
- *Downey Type II*: larger in appearance with moderately clumped chromatin, abundant cytoplasm, with a gray-blue color and the characteristic peripheral intensification of the basophilia, sometimes containing a few azurophilic granules, easily deformed by the surrounding red blood cells.
- *Downey Type III*: similar to immunoblasts with even more abundant cytoplasm, with similar staining characteristics and a more immature nucleus, irregular shape, sometimes trapezoidal or notched, with reticular chromatin and the presence of nucleoli.

Moreover, some authors call attention to the fact that “Clover Leaf” nuclei are often observed for this type of disease—large lymphocytes with abundant basophilic cytoplasm, some with large central nucleoli, similar to immunoblasts with a four-leaf clover nucleus (8).

However, the “clue” for making a differential diagnosis between mononucleosis and other forms of lymphocytosis appears to be represented by the presence, in the first case, of apoptotic lymphocytes, morphologically characterized by loss of volume, condensation of the chromatin along the nuclear membrane with intensely basophilic coloration, and/or nuclear fragmentation in spherical structures surrounded by membranes containing condensed chromatin (9–11) (Fig. 1).

All hematological analyzers are capable of separating leukocyte populations based on the size, complexity, and cellular content, and therefore highlight and report the presence of lymphocytes with characteristics of atypia (12–14). However, this flag is not necessarily attributable to the presence of Downey-type lymphocytes, and therefore to mononucleosis syndrome, as it can also be present

in other pathological conditions such as other types of reactive lymphocytosis or neoplastic lymphocytosis (typical chronic lymphocytic leukemia (CLL) and atypical CLL, leukemic phase non-Hodgkin lymphoma (NHL), acute lymphoblastic leukemia (ALL)). The element characteristic of mononucleosis syndrome, which is not found in clonal and nonclonal diseases other than this, is instead represented by the presence of apoptotic lymphocytes. These are T CD8⁺ lymphocytes responsible for cellular killing, which in the primary infection, express the isoforms CD45RO, human leukocyte antigen-antigen D related (HLA-DR), and CD38, expand and remain insensitive to apoptotic stimuli due to the higher expression of Bcl-2 that makes them resistant to Fas ligand (FasL). With the progress of the disease, and therefore with the reduction of viremia, because of a system of self-modulation, there is a reduction of Bcl-2 levels, for which these cells develop sensitivity for FasL and therefore undergo apoptosis in order to restore immune homeostasis (15, 16).

A characteristic aspect of these CD45RO⁺ lymphocytes is represented by the fact that they spontaneously and quickly die of apoptosis when they are removed from patients and incubated in vitro, whereby the portion of apoptotic lymphocytes, in samples that have just been withdrawn, is lower as compared to the samples that can be found after a few hours (10).

Cellular degeneration leads to variations in size and complexity, which leads to a different position for this population in hematological analyzers, in relation to the forward scatter (FSC) and side scatter (SSC), as compared to normal and atypical lymphocyte populations. Based on this concept, Mentz et al. (17) in 1998 and then Taga et al. (9) in 2000 evaluated, respectively, the ability of the Bayer H1 (Bayer Corporation, Terrytown, New York, USA) and Coulter MAXM (Coulter Corp., Miami, FL) analyzers to identify the presence of apoptotic cells in peripheral blood samples. Moreover, Taga et al. (10), using the Coulter MAXM analyzer, demonstrated the capacity to differentiate subjects with mononucleosis syndrome with an atypical presentation based on the presence of apoptotic lymphocytes.

Aim of the Study

Sysmex XN-1000 (Sysmex Co., Japan) is a new-generation hematological analyzer designed to improve both accuracy of cell counting in cytopenic samples as well as specificity of flags as regards the presence of atypical cellular populations (18, 19). To this end, it uses new analytical channels including white cell differential channel (WDF) capable of providing the extended leukocyte formula to six populations, separating lymphocytes, monocytes, eosinophils, basophils, neutrophils, and immature granulocytes; white cell nucleated channel (WNR)

for counting WBC, nucleated red blood cells (NRBC) and basophils; white cell precursor channel (WPC) used for performing reflex tests only on samples flagged in the WDF channel for the presence of blasts or abnormal lymphocytes. Analysis of the three WNR, WDF, and WPC channels is performed by fluorescence flow cytometry with 633-nm laser light, after treatment of the cells with lysing reagents and specific polymethine-based fluorescent dyes that bind to the nucleic acids and cytoplasmic organelles (20). In particular, in the WDF channel, a surfactant causes hemolysis and the dissolution of erythrocytes (RBC) and platelets (PLT), while it changes the membrane of WBCs in a differential manner; therefore, the fluorescent dye enters the cells and stains their nucleic acids. The intensity of the signal in relation to FSC, SSC, and side fluorescence (SFL) allows the leukocyte populations to be separated into well-defined clusters, based on the size (FSC), internal complexity (SSC), and content in nucleic acids, DNA and RNA (SFL), respectively. The data obtained are thus expressed through two bidimensional (2D) dot plots: WDF plot, SFL versus SSC (SFL-SSC); and WDF plot, SSC versus FSC (SSC-FSC).

Analyzing samples from normal subjects as well as patients with various forms of lymphocytosis, it has been observed that in patients with mononucleosis syndrome, the SFL-SSC dot plot presented, in addition to a share of atypical lymphocytes—which can be distinguished through the increase in fluorescence—another population that is distinct from samples containing normal lymphocytes and is characterized by increased complexity (SSC). The population in question is also made visible on the SSC-FSC dot plot due to the increased complexity and reduced volume as compared to the normal lymphocyte population (Fig. 2a–d).

Furthermore, the aforementioned population was not found in other types of analyzed samples. As the cytographic data were compatible with the morphological characteristics of apoptotic lymphocytes, it was hypothesized that the cluster in question could be linked to the same cellular population. The scope of work was therefore to select the unknown population in order to quantize it and then check if the numerical data are comparable to the number of apoptotic lymphocytes found on the peripheral blood smear, in order to confirm the nature of the abnormal cluster by characteristics of FSC, SSC, and SFL.

Furthermore, it was observed that the presence of the population of apoptotic lymphocytes causes a dilation of the lymphocyte cluster on the WDF cytogram; therefore, the numerical data of the structural parameters supplied by Sysmex XN-1000 relative to the width of distribution of lymphocyte cluster parameters volume (LY-WZ = lymphocyte – volume distribution width), complexity (LY-WX = lymphocyte – complexity distribution width), and fluorescence (LY-WY = lymphocyte – fluorescence dis-

Fig. 2. SFL-SSC (left panel) and SSC-FSC (right panel) dot-plot WDF channel Sysmex XN-1000 related to normal sample (a), morphologically typical CLL (b), leukemic phase mantle (NHL) (c), and mononucleosis syndrome (d).

tribution width) were used in order to create an algorithm that allowed a differential diagnosis between mononucleosis syndrome and other hematological diseases.

MATERIALS AND METHODS

A total of 698 samples taken from normal routine and urgent care were analyzed, of which 39 were taken from 13 patients with IM (ten of whom were serologically positive for EBV and 3 for CMV) at first diagnosis and in follow-up within 7 days; 76 samples were taken from patients affected by chronic lymphoproliferative diseases in follow-up or at first diagnosis performed in observance of the WHO 2008 document (21); 25 samples were taken from patients with relative or absolute, reactive non-clonal lymphocytosis; 40 samples were taken from healthy

Fig. 3. Gating strategy performed on a peripheral blood sample in a patient affected by Mononucleosis Syndrome. In (a) On dot-plot Side Fluorescence Signal vs Side Scatter Signal gating of the leukocyte population R0 = Eosinophils, R1 = neutrophils, R2 = Monocytes, R3 = Lymphocytes, R4 = Noise, R6 = NRBC; in (a) and (b) On dot-plot Forward Scatter Signal vs Side Scatter Signal identification of the cluster of apoptotic lymphocytes (Ly-Apopt).

donors. Furthermore, 518 samples from subjects affected by other hematological and nonhematological diseases or subjects with known interferences (lipemic samples, hemolyzed samples, samples taken over 24 h earlier with cryoagglutinin and cryoglobulin, piastrinic aggregation, etc.) were also evaluated (myelodysplastic syndrome diseases (MDS), myeloproliferative disorder (MPD), MDS–MPD, acute myeloid leukemia (AML), ALL, etc., and reactive neutrophilias, neoplasia). All samples were collected in K3-EDTA and processed within nonstandardized intervals of time, but in all cases not more than 8 h from the time of withdrawal (hemochromocytometric exam through Sysmex XN-1000, and performance of the smear for subsequent May Grünwald-Giemsa staining and 500 cell count by a single expert morphologist).

Finally, we used 40,000 files derived from an internal database Sysmex-Dasit to test and validate our algorithm. As in our case, 40,000 samples were collected in K3-EDTA, processed within nonstandardized interval of time without refrigeration.

Methods

The Sysmex XN-1000 beyond numerical data of CBC provides information about the positional parameters for erythrocyte and leukocyte populations. For each leukocyte population (neutrophils, lymphocytes, monocytes, eosinophils, and basophils), the media and distribution width of the cluster can be obtained. These parameters can be viewed directly on the screen or can be saved in a text file.

The Sysmex XN-1000 analyzer can save data relative to the samples analyzed in the flow cytometry standard (FCS) format for analysis through dedicated software. For each sample, files related to the WPC, WNR, WDF, reticulocyte (RET), PLT-F channels are generated in which, with the respective parameters (SSC, SFL, and FSC), bi-parametric analyses can be performed as regards the populations of WBC, RBC, and PLT. In this work, Weasel version 3.1 software was used (Walter and Eliza-Institute, Melbourne, Australia) in order to analyze the files, and the files generated for the WDF channel were analyzed.

The gating strategy provided

- (1) gating of the leukocyte populations in the SFL–SSC dot plot (Fig. 3a);
- (2) gating of the presumed population of apoptotic lymphocytes in the SSC–FSC and SFL–SSC dot plots (Fig. 3b).

Therefore, the percentage of events was detected in the gates of the presumed population of apoptotic lymphocytes (Ly-Apopt) in the total of WBC, and the correlation between events in the two Ly-Apopt gates. The count under the optical microscope was performed on 500 cells on the peripheral blood smear stained using the May Grünwald-Giemsa method, which also includes apoptotic lymphocytes in the differential formula.

Statistical Analysis

The statistical analysis was performed by MedCalc software version 11.4.2.0 (MedCalc, Ghent, Belgium).

Fig. 4. Correlation between %Ly-Apopt of the SSC-FSC cluster of the WDF channel and the count in morphological review. (a) Passing and Bablok linear regression; (b) Bland and Altman difference plot.

Comparison between groups was performed by analyzing the Box and Whiskers distribution graphs; data were not distributed normally, which was confirmed by the D'Agostino-Pearson P -value <0.05 , and were presented as median, minimum-maximum, and interquartile range (IQR). The Passing and Bablok regression and

Spearman's test were used for analyzing correlation and association (a " P " value lower than 0.05 was considered to be statistically significant). The differentiating capacity related to the parameters being analyzed was evaluated through area under the curve (AUC) for the receiver operating characteristic (ROC) curve.

Fig. 5. Correlation between %Ly-Apopt of the SFL-SSC cluster of the WDF channel and the count in morphological review. (a) Passing and Bablok linear regression; (b) Bland and Altman difference plot.

Fig. 6. Correlation between %Ly-Apopt of the cluster in the physical SSC–FSC gate and %Ly-Apopt of the cluster of the gate in SFL–SSC fluorescence. (a) Passing and Bablok linear regression; (b) Bland and Altman difference plot.

RESULTS

Correlation of %Events in the “Ly-Apopt” Cluster Versus Apoptotic Count Under Optical Microscopy

The analysis performed through the Passing and Bablok regression showed an excellent correlation between the percentage of “Ly-Apopt” events observed in the SSC–FSC gate and SFL–SSC gate found in the WDF channel and percentage of apoptotic lymphocytes observed with the differential count on the peripheral blood smear ($y = 1.0x - 0.7$, $R^2 = 0.79$ and $y = 1.02x + 0.01$, $R^2 = 0.753$, respectively) (Figs. 4a and 5a).

Analyzing the correlation performed through Bland and Altman difference plots, neither proportional errors were inferred (over- or underestimate) nor periodic trends or nonuniformity of variance were observed even for high values, therefore showing good agreement with the manual method (mean $\pm$ 2SD %Ly_ApoptSFL vs. blood smear and %Ly_ApoptSFL vs. blood 0.1 ± 9.0 and 0.9 ± 8.5 , respectively) (Figs. 4b and 5b).

Finally, the percentage value of events in the %Ly-Apopt physical gate (SSC–FSC) perfectly correlated to the value observed in the fluorescence gate (SFL–SSC) ($y = 0.99x + 0.91$; $R^2 = 0.99$) (Fig. 6). In Bland and Altman difference plots (Fig. 6b), neither proportional errors were inferred (over- or underestimate) nor periodic trends or nonuniformity of variance were observed (mean $\pm$ 2SD %Ly_ApoptSFL vs. %Ly_ApoptSFL 0.3 ± 2.5).

Evaluation of Events in the “Ly-Apopt” Box

Calculation of the percentage of events in the “Ly-Apopt” box shows that this percentage is significantly increased in mononucleosis syndrome (samples with IM, $n = 39$; median 7.25; min–max (1.3–19.45); IQR 8.4; $P < 0.05$) both as compared to normal samples (samples $n = 40$; median 0.34; min–max (0.13–0.75); IQR 0.12) and

clonal lymphocytosis (samples $n = 76$; median 0.24; min–max (0.0–10.0); IQR 0.28) and nonclonal lymphocytosis (samples $n = 25$; median 0.29; min–max (0.15–0.80); IQR 0.19), as well as compared to all other conditions analyzed (samples $n = 518$; median 0.21; min–max (0.0–3.38); IQR 0.24) (Fig. 7 and Table 1).

Considering the increased statistical significance of the percentage value of events in the Ly-Apopt SSC–FSC clusters, a possible differentiating value was evaluated through the ROC curve. Thus, it was observed that using a cut-off over 1.2%, one could obtain an AUC = 0.997 (95% CI: 0.983–1.000) with 100% sensitivity (95% CI sensitivity was 91.6–100) and 98% specificity (95% CI specificity was 95.0–99.5) (Fig. 8a and b).

Similarly, a high AUC equal ($P < 0.05$) to 0.990 (95% CI: 0.968–0.998) with 100% sensitivity (95% CI sensitivity was 91.6–100) and, although slightly lower, 94.1% specificity (95% CI specificity was 89.9–96.9) was also observed for the Ly-Apopt SFL–SSC cluster (Fig. 9a and b).

Evaluation of the Range of Distribution of LY-WX, LY-WY, LY-WZ Parameters in the Lymphocyte Cluster

In IM, as observed by the comparison of Box and Whiskers distributions with normal samples and other diseases, one notes a significant variation at the expense of the range of all three LY-WX, LY-WY, LY-WZ positional parameters of the lymphocyte cluster (Fig. 10a–c and Table 1). This particularly concerns physical parameters, as the lymphocyte population is “morphologically complex” and has strongly heterogeneous dimensions. Based on these data, therefore, the AUC was evaluated for each individual parameter (Fig. 11).

For a simultaneous increase, above the cut-off values, LY-WX > 500 and LY-WY > 1,000 and LY-WZ > 700, 99% specificity and 100% sensitivity was observed

TABLE 1. Obtained Value for SSC, SFL, and FSC Cell Population Data in Normal Subjects (CTR), Clonal Lymphocytosis (CLL/aCLL/NHL), Patient With Infectious Mononucleosis, Reactive Lymphocytosis, and All Disease

	CTR	CLL/aCLL/NHL	Infectious mononucleosis	Reactive lymphocytosis	All Disease
%Ly-Apopt	0.34; (0.13–0.75); 0.12	0.24; (0.0–10.0); 0.28	7.25; (1.3–19.45); 8.4; * $P < 0.0001$	0.29; (0.15–0.80); 0.19	0.21; (0.0–3.38); 0.24
LY-WX	480.5; (408–574); 61.0	503; (330–669); 102.0	739; (519–988); 213.2 * $P < 0.0001$	519; (417–1,238); 140.2	501; (26–1,238); 84.0
LY-WY	880; (790–1,137); 99.0	934.5; (585–1,556); 269.0	1,612; (1,181–2,096); 554.5; * $P < 0.0001$	847; (739–1,871); 306.5	904; (54–3,512); 200.0
LY-WZ	420.5; (383–466); 20.0	438; (320–940); 64.5	898; (760–1,175); 221.3; * $P < 0.0001$	426; (364–1,375); 66.0	446; (61–1,375); 87.5

Data are given as median; min–max; IQR.

*P-value is referred to the comparison of %Ly-Apopt, SSC, SFL, or FSC value in CTR, CLL/aCLL/NHL, infectious mononucleosis and reactive lymphocytosis, and All Disease.

Fig. 7. Box and Whiskers plot related to the distribution of the percentage of events in the Ly-Apopt cluster in: controls (CTR), subjects with clonal lymphoproliferative diseases (CLL-aCLL-NHL), infectious mononucleosis (IM), relative lymphocytosis or absolute nonclonal lymphocytosis (Lymph-react), other hematological and nonhematological diseases (All Disease).

(Fig. 12). However, the specificity reached 100%, rendering the presence of an erythroblast count (NRBC) $< 0.03 \times 10^3/\mu\text{L}$ —a further differentiating parameter.

Validation and Revaluation of the Diagnostic Algorithm in Relation to a High Number of Samples

Applying the algorithm to a higher number of cases ($N = 40,000$), a 5% increase was observed in microscopic revisions that did not correlate with the expected incidence of mononucleosis syndrome. Reevaluating the FCS files related to these samples, in 2.5% of them, the flags for atypical lymphocytes (Atypical Lympho) were not found, while the Ly-Apopt share on the SSC–FSC and SFL–SSC dot plots was $< 1.2\%$. Therefore, the ideal parameters for revision of the algorithm should be strictly correlated with the etiopathogenesis and classic morphology of mononucleosis syndrome, and therefore satisfy the requirements of sensitivity and specificity related to the presence of large lymphocytes of mononucleosis (Downey) and apoptotic degenerative shapes.

The algorithm was therefore re-elaborated, including the further differentiating parameters, “Atypical Lympho” flag, and simultaneous presence of a number of events $> 1.2\%$ in the “Ly-Apopt” box on the SSC–FSC and SFL–SSC dot plots. All parameters examined were

therefore taken with a cut-off in order to maximize sensitivity and, at the same time, their combined use entailed a high specificity.

Therefore, only the physical parameters of the lymphocyte clusters provided a sure indication concerning the presence of Downey lymphocytes, or

- presence of “Atypical Lympho” flag,
- LY-WX > 500 ,
- LY-WY $> 1,000$,
- LY-WZ > 700 ,

while the increase in complexity of the LY-WX lymphocyte cluster was simultaneously examined for the presence of apoptotic lymphocytes on both physical Ly-Apopt-FSC gate as well as Ly-Apopt-SFL fluorescent gate, excluding cases with the presence of erythroblasts in a circle:

- NRBC $< 0.03 \times 10^3/\mu\text{L}$.

However, some cases of leukemic phase myeloma provided false positives, for which the percentage of apoptotic lymphocytes on both gates was also added:

- LY-WX > 500 ,
- LY-WY $> 1,000$,
- LY-WZ > 700 ,

Fig. 8. (a) ROC curve for %Ly-Apopt of the SSC–FSC cluster in subjects with hematological and nonhematological diseases. The sensitivity was 100% and specificity was 98%, AUC = 0.997. (b) Interactive point diagram between all diseases (0 = All Disease) and the infectious mononucleosis group (1 = IM).

Fig. 9. (a) ROC curve for %Ly-Apopt of the SFL-SSC cluster in subjects with hematological and nonhematological diseases. The sensitivity was 100% and specificity was 94.1%, AUC = 0.990. (b) Interactive point diagram between all diseases (0 = All Disease) and the infectious mononucleosis group (1 = IM).

Fig. 10. Box and Whiskers distribution of structural parameters related to the range of the lymphocyte cluster in (a) LY-WX (SSC); (b) LY-WY (SFL); (c) LY-WZ (FSC) in normal subjects (CTR), in subjects with CLPD (CLL/aCLL/NHL), in subjects with infectious mononucleosis (Mono), in subjects with reactive lymphocytosis (Lympho_React) and in other diseases (All_Disease).

- %Ly-Apopt SSC–FSC > 1.2%,
- %Ly-Apopt SFL–SSC > 1.2%,
- NRBC < $0.03 \times 10^3/\mu\text{l}$.

All the designated rules were simultaneously satisfied, reaching a specificity of 100%.

DISCUSSION AND CONCLUSIONS

IM is a clinical syndrome commonly associated with primary infection by the EBV. Seroepidemiological surveys estimate that 95% of the global adult population has contracted the EBV infection, while the total incidence of the disease in the United States is 500 cases per

Fig. 10. Continued.

Fig. 11. ROC curve of structural parameters related to the range of the lymphocyte clusters LY-WX (SSC), LY-WY (SFL), and LY-WZ (FSC) among subjects with infectious mononucleosis and other diseases (LY-WX: AUC = 0.911, sensitivity = 81.8%, specificity = 95.0%; LY-WY: AUC = 0.982, sensitivity = 100%, specificity = 93.1%; LY-WZ: AUC = 0.974, sensitivity = 100%, specificity = 97%).

Fig. 12. ROC curve for values of structural parameters related to the range of the lymphocyte cluster contextually greater than the following cut-offs: LY-WX (SSC) > 500, LY-W (SFL) > 1,000, LY-W (FSC) > 700 among subjects with infectious mononucleosis (IM) and other diseases (All Disease); AUC = 0.998, sensitivity = 100%, specificity = 99%.

100,000 residents per year. Sore throat, general malaise, and asthenia are the most common symptoms presented; pharyngitis, fever, and lymphadenopathy represent the classic semiologic triad. However, this pathological condition can often occur without the common clinical changes and typical hematological signs, making its identification difficult.

Conversely, in some circumstances, a lymphocytosis with an increase in the percentage of atypical lymphocytes may generate diagnostic confusion with lymphoproliferative diseases. This may therefore make it problematic, especially in emergency regimes, to correctly profile the patient and requires performance of several, and not always economical, diagnostic tests. A study performed by Borer et al. (19) in 1999 showed that in the United States, the costs related to unnecessary diagnostic procedures and medical treatment for management of patients with mononucleosis syndrome of an atypical presentation reached \$12,000 per patient. Considering the incidence of the pathology and percentage of cases with atypical clinical presentations, which, based on a study performed by Taga et al. (10), reaches about 60%, it is easy to understand how rapid diagnostic tests are useful. Even in our study (data not shown), 9 of 13 patients were hospitalized in nonappropriate departments or referred to a general practitioner, and inappropriate further diagnostic tests (radiological and laboratory) were requested for all of them.

This study shows that the Sysmex XN-1000 hematological analyzer makes it possible to quickly differentiate between samples of subjects affected by mononucleosis syndrome as compared to other pathological conditions. First and foremost, the analyzer provides useful and immediate cytographic data represented, only in mononucleosis syndrome, by the presence of a cellular cluster characterized by a reduced volume (FSC) and increased complexity (SSC) on the SSC versus FSC dot plot, as compared to the normal lymphocyte population. Furthermore, this population is also evident on the SFL-SSC dot plot, especially due to its increased complexity as compared to the normal lymphocyte population. Due to its cytographic characteristics, the aforementioned cluster can be attributed to the presence of apoptotic lymphocytes. Therefore, considering the high diagnostic value of this population in mononucleosis syndrome, its nature was confirmed through gating on the FCS file, quantization and comparison of the classic morphological data, with an excellent correlation. However, one outlier could be observed (Figs. 4 and 5) because we collected the sample in nonstandardized interval of time, so, for this sample, we made the peripheral blood smear just as the sample arrived in the laboratory, and performed CBC after about 8 h: for this reason it would be ideal for standardization of the procedure to collect sample,

however this is not possible in routine and urgency hospital laboratory.

Even in light of its undeniable usefulness, cytographic data may not be sufficient for identifying patients with mononucleosis syndrome and its differentiation as compared to other pathologies, particularly in contexts in which heavy workloads do not allow the evaluation of dot plots. Based on these factors, this study suggests the implementation of an algorithm that, integrating the data related to the "Atypical Lympho" flag, increase in the distribution ranges of the lymphocyte cluster (LY-WX, LY-WY, LY-WZ), and presence of events in an area of the FCS-SSC and SFL-SSC dot plots not occupied by normal lymphocytes or by atypical lymphocytes, may generate a "mononucleosis" alarm. Not including, among other elements, the data related to the absolute lymphocyte count, the algorithm also allows detection of mononucleosis syndrome without absolute lymphocytosis (2/13 of our patients).

The results of our study should be confirmed with a higher number of cases of mononucleosis syndrome and other diseases, taking into consideration the different standardizations of analyzers in various laboratories, and the data obtained must refer to samples analyzed not immediately after withdrawal, given the nature of CD45RO+ lymphocytes.

REFERENCES

1. Sprunt TP, Evans FA. Mononuclear leukocytosis in reaction to acute infections. *Bull Johns Hopkins Hosp* 1920;31:410-417
2. Epstein MA, Achong BG, Barr MY. Virus particles in cultured lymphoblasts from Burkitt's lymphoma *Lancet* 1964;i:702-703
3. Heath CW Jr, Brodsky AI, Potolsky AI. Infectious mononucleosis in a general population. *Am J Epidemiol* 1972;95(1):46-52.
4. Sumaya CV, Ench Y. Childhood Infectious mononucleosis-like illness not associated with Epstein-Barr virus. *Pediatr Res* 1987;21:336A.
5. Hoagland J, Colonel RMC. The transmission of infectious mononucleosis. *Am J Med Sci* 1955;229:262-272.
6. Downey H, McKinley CA. Acute lymphadenitis compared with acute lymphatic leukemia. *Arch Intern Med* 1923;32:82-112.
7. D'Onofrio G, Zini G. *Morfologia del Sangue*. Roma: Verduci Editore; 1997. p 18.
8. Inoue S. Clover leaf nucleus of atypical lymphocytes in infectious mononucleosis. *Br J Haematol* 1988;70:381-338.
9. Taga K, Yoshida M, Kaneko M, et al. Contribution of automated hematology analysis to the detection of apoptosis in peripheral blood lymphocytes. *Citometry* 2000;42:209-214
10. Taga K, Taga H, Tosato G. Diagnosis of atypical cases of infectious mononucleosis. *Clin Infect Dis* 2001;33:83-88
11. Kulkarni JD. Apoptotic lymphocyte as clue to infectious mononucleosis. *Blood* 2012;120(4):708.
12. Hotton J, Broothaers J, Swaelens C, Cantinieaux B. Performance and abnormal cell flagging comparisons of three automated blood cell counters. *Am J Clin Pathol* 2010;140(6):845-852.

13. Kang S-H, Kim HK, Ham CK, Lee DS, Cho HI. Comparison of four hematology analyzers, Cell Dyn Sapphire, ADVIA 120, Coulter LH 750 and Sysmex XE-2100, in terms of clinical usefulness. *Int J Lab Hematol* 2008;30:480–486.
14. Briggs C, Linssen J, Longair I, Machin SJ. Improved flagging rates on the Sysmex XE 5000 compared with the XE 2100 reduce the number of manual film review and increases laboratory productivity. *Am J Clin Pathol* 2011;136(2):309–316.
15. Uehara T, Miyawaki T, Ohta K, Tamaru Y, Yokoi T, Nakamura S, Taniguchi N. Apoptotic death of primed Cd45RO+ T lymphocytes in Epstein–Barr virus-induced infectious mononucleosis. *Blood* 1992;80:452–458.
16. Tanner and Alfieri. Epstein–Barr virus induces Fas(CD95) in T cells and Fas ligand in B cells leading to T-cell apoptosis. *Blood* 1999;194(10):3439–3447.
17. Mentz F, Baudet S, Blanc C, Issaly F, Binet JL, Merle-Beral H. Simple, fast method of detection of apoptosis in lymphoid cells. *Cytometry* 1998;32:95–101.
18. Kono et al. United States Patent Application Publication. 2012;US2012/0115159 A1.
19. Borer A, Gilad J, Haikin H, Reisenberg K, Porath A, Schlaeffer F. Clinical features and costs of care for hospitalized adults with primary Epstein–Barr virus infection. *Am J Med* 1999;107:144–148.
20. Nadine SJ, Yihui C, Penny J, Tymish YO, Manivannan E, Maged H, Lucjan S, Ravindra KP. Evaluation of polymethine dyes as potential probes for near infrared fluorescence imaging of tumors: Part – 1. *Theranostics* 2013;3(9):692–702.
21. WHO 2008 Classification of Tumours of Haematopoietic and Lymphoid Tissues, fourth edition.
22. Heath CW Jr, Brodsky AI, Potolsky AI. Infectious mononucleosis in a general population. *Am J Epidemiol* 1972;95(1):46–52.