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Biomarkers involved in evaluation of platelets function in South-Eastern Romanian patients with hematological malignancies subtypes

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Abstract

At present, various researches presented how subtypes of hematological malignancies are related to stages of the immune response, because the activated immune system represents a promising form in cancer treatment. This study explores the relationship between the adaptive immune system (T cells), and the coagulation system (platelets, platelet membrane glycoproteins, platelets derivate microparticles) which seems to play an important role in host immune defense of patients with acute myeloblastic leukemia (AML) or B cell lymphoma (BCL), 2 of the most common hematological malignancies subtypes.

Blood samples (n = 114) obtained from patients with AML or BCL were analyzed for platelet membrane glycoproteins (CD42b, CD61), glycoprotein found on the surface of the T helper cells (CD4$^+$), protein complex-specific antigen for T cells (CD3$^+$), platelet-derived microparticles (CD61 PMP) biomarkers by flow cytometry, and hematological parameters were quantified by usual methods.

In patients with AML, the means of the percentage of the expressions of the molecules on platelet surfaces (CD61 and CD42b, $P < .01$; paired T test) were lower as compared to both control subgroups. The expression of cytoplasmic granules content (CD61 PMP) had a significantly higher value in patients with AML reported to controlling subgroups ($P < .01$; paired T test), which is suggesting an intravascular activation of platelets.

The platelet activation status was presented in patients with low stage BCL because CD61 and CD42b expressions were significantly higher than control subgroups, but the expression of CD 61 PMP had a significantly decreased value reported to control subgroups (all $P < .01$; paired T test). T helper/inducer lineage CD4$^+$ and T lymphoid lineage CD3$^+$ expressions presented significant differences between patients with AML or low stage BCL reported to control subgroups (all $P < .01$; paired T test).

Platelet–lymphocyte interactions are involved in malignant disorders, and CD61, CD42b present on platelet membranes, as functionally active surface receptors mediate the adhesion of active platelets to lymphocytes, endothelial cells, and cancer cells.

Abbreviations: AML = acute myeloblastic leukemia, BCL = B cell lymphoma, CD3$^+$ = protein complex-specific antigen for T cells, CD4$^+$ = glycoprotein found on the surface of the T helper cells, CD42b, CD61 = platelet membrane glycoproteins, CD61 PMP = platelet-derived microparticles.

Keywords: acute myeloblastic leukemia, B cell lymphoma, CD3$^+$, CD4$^+$, CD42b, CD61, CD61 PMP, platelets

1. Introduction

Acute myeloblastic leukemia (AML) is characterized by the clonal proliferation of malignant hematopoietic precursor cells in the hematogenous marrow. Malignant cell accumulation in bone marrow results in dissociation of normal hematopoiesis by suppressing the growth and differentiation of normal bone marrow cells and decreasing blood cell production. In consequence, in the peripheral blood appear variable cytopenias associated with clinical manifestations such as anemia, infections, and hemorrhagic syndromes. Leukemic cells can also invade extramedullary tissues such as the meninges, gonad, liver, spleen, and lymph nodes.[1,2]

Malignant lymphoma is part of a heterogeneous tumor group of the lymphoid system, which can involve any organ or tissue. Lymphoma is divided into 2 categories according to World Health Organization (WHO) classification: Hodgkin lymphoma and non-Hodgkin lymphoma.[3] Non-Hodgkin lymphoma is a malignancy characterized by a monoclonal proliferation of B cell or, rarely, of T/NK-cell.[4] Clinically, non-Hodgkin lymphoma is classified as indolent, aggressive, or extremely aggressive malignancy.[5,6]

Leukemia has an incidence rate of 2.1% (1724 new cases/year) in the Romanian adult population. For non-Hodgkin lymphoma, WHO[7] published an incidence rate of 1.7% (1424 new cases/year). AML is the most common type of acute leukemia. The average age at diagnosis is 65 years and the incidence increases with age.[8]

Immunophenotyping by CD-biomarkers expressions with flow cytometry is useful in the diagnoses of the acute myeloblastic leukemia (AML)/B cell lymphoma (BCL). Associated CD antigens of the leukocytes are molecules involved in functions such as cell-to-cell interactions, cytokine receptors, cell signaling, ion channels, transporters, enzymes, immunoglobulins, and adhesion molecules.[9] Effector T lymphocytes exhibit antitumor activity in the tumor, and their intratumoral presence increases the survival rate of these patients. Regulatory T lymphocytes and macrophages generate an immunosuppressive environment that counteracts antitumor immunity, promotes tumor progression, and decreases the patient's survival rate.[10–12]

Bleeding is known to occur during diagnosis and chemotherapy, being another important factor threatening the lives of AML patients. The reduction of platelets number is a determinant to assess the bleeding risk. Patients with a low platelet count, who were diagnosed and treated for a long period of time, presented a small amount of bleeding. The bleeding is associated with platelet dysfunction, not only with the platelet number,[13] and the level of platelet membrane glycoproteins and the expression of the platelet membrane glycoproteins can be analyzed by flow cytometry to assess the platelet function.[14,15] Recently, platelet-derived microparticles (PMPs) are employed as an alternative evaluation of platelet activation. PMPs induce the apoptosis of effector T cells [16–19] and induce T lymphocytes regulatory responses.[20,21]

PMPs that are shed from platelet surface membranes constitute the majority of circulating microparticles, being implicated in procoagulant, pro-inflammatory, and pro-atherosclerotic actions. The diagnostic value of microparticles was investigated in patients with different cancer types, including bladder cancer,[22] prostate cancer,[23] and colorectal cancer.[24]

In our study, the platelets functions in correlation with T lymphocytes count were assessed, by analyzing the platelets membranes glycoproteins expressions (CD61, CD42b), platelets derive microparticles (CD61 PMP), T lymphoid lineage (protein complex-specific antigen for T cells [CD3+]), and T helper/inducer lineage (glycoprotein found on the surface of the T helper cells [CD4+]) by flow cytometry. Associated parameters such as total platelets, leukocytes, and lymphocytes counts, made by an automated hematologic analyzer, were also analyzed.

2. Materials and methods

2.1. Case selection

All blood samples (n=114) were obtained from patients (who signed informed consent forms, agreeing to participate in this study), which are treated in the Hematology Department of Sf. Apostol Andrei Clinical Emergency County Hospital in Constanta, Romania. Blood samples were analyzed for CD-biomarkers by flow cytometry and hematological determinations, at the Cell Biology Department of Centre for Research and Development of Morphological and Genetic Studies of Malignant Pathology, *Ovidius* University of Constanta, Romania.

In agreement with WHO classifications, the patients were divided into 2 groups:

1. Patients with AML were, under treatment, divided into 2 subgroups by age (≤65 years, n=15 and >65 years, n=14), and a control group of match healthy subjects was assigned for each subgroup (n=15, ≤65 years and n=14, >65 years).
2. Patients with BCL, under treatment, divided into 2 subgroups by Ann Arbor stage:
   - patients with Ann Arbor I–II tumoral stages (n=16), reported to match control healthy patients (n=16);
   - patients with Ann Arbor III–IV tumoral stages (n=12), reported to match control healthy patients (n=12).

2.2. Reagents and equipment

The flow cytometry (Attune, Acoustic focusing cytometer, Life Technologies) was used to measure the expressions of CD61, CD42b, CD3+, and CD4+. Anti-CD42b-PE (HIP1) and anti-CD61-PE (integrin beta 3, Invitrogen, eBioscience) monoclonal antibodies conjugated with phycoerythrin, were used to assess platelet expressions of GPIbα and GPIIIa. Alexa Fluor 488 conjugated monoclonal antibodies to CD3+, CD4+ (anti-CD3-Alexa Fluor 488, anti-CD4-Alexa Fluor 488, Invitrogen, Molecular Probes) were used to determine T lymphoid and T helper/inducer cells from blood samples. For negative controls, mouse IgG (Invitrogen, eBioscience) were used.

Before analysis of CD61 PMP, the flow cytometer was first set by using fluorescent beads (Attune performance tracking beads, Labelling and detection, Life technologies), with standard size (4 intensity levels of beads population), and the quantity was established by enumerating PMPs below 1 μm. The number of CD61 PMP was calculated based on the event count from the bead tube collected. PMPs were gated by Forward Scatter and Side Scatter parameters, and then identified with CD61-PE. For data collection, graphics by flow cytometry were used with Attune Cytometric Software v.1.2.5, Applied Biosystems, 2010. Pentra XLR automated blood cell analyzer (Horiba Medical, Diamedix, Romania) was used for determinations of the total number of platelet (PLT), leukocytes, and lymphocytes counts.

2.3. CD61, CD42 B platelet membrane glycoproteins, and CD3$^+$, CD4$^+$ T lymphocytes detections by flow cytometry dual stain

The blood samples were collected into tubes (4 mL) with anticoagulant EDTA-K2. All measurements were performed within 120 minutes after blood withdrawal.

Flow cytometry tubes were divided into 3 categories:

1. samples with CD61-PE and CD4$^+$-Alexa Flour 488 dual stain;
2. samples with CD42b-PE and CD3$^+$-Alexa Fluor 488 dual stain;
3. samples with control negative-IgG stain.

In tubes of flow cytometry were introduced 100 μL blood sample, 5 μL of CD61-PE, and 10 μL of CD4$^+$-Alexa Flour 488. The contents were gently vortexed and incubated into darkness, for 25 minutes at 37°C. 100 μL blood sample, 5 μL of CD42b-PE, and 10 μL of CD3$^+$-Alexa Fluor 488 were added into other tubes, vortexed, and incubated into darkness, for 25 minutes at 37°C. For each sample, a control tube with 100 μL blood sample and 5 μL of the negative control (mouse IgG) were made, and the tubes were vortexed and incubated into darkness, for 25 minutes at 37°C. Three milliliters of flow cytometry stain buffer were added into each tube, and vortexed for 1 minute before analysis.

Platelets were identified by flow cytometry based on size and platelet specific CD61 and CD42b surface expressions. T helper/inducer lymphocytes and total T lymphocytes (thymocytes, T lymphocytes, and NK cells) were determined by flow cytometry based on size and lymphocytes specific CD4$^+$ and CD3$^+$ monoclonal antibodies expressions.

2.4. Platelet-derived microparticles-CD61 determination by flow cytometry

Collected blood samples were centrifuged at 10,000× g for 10 minutes. From obtained plasma samples, were aspirated 100 μL of plasma and introduced into flow cytometer tubes. Five microliters of CD61-PE were added over the plasma samples, and tubes were vortexed and incubated into darkness, for 25 minutes

at 37°C. Two milliliters of flow cytometry stain buffer were added into each tube and were vortexed for 1 minute before analysis at equipment. In parallel, were worked control plasma samples, made from 100 μL plasma and 5 μL of the negative control (mouse IgG), which followed the same steps mentioned before.

### 2.5. Statistical analysis

Obtained results were presented as mean values with standard errors for CD61, CD42b platelets, CD3$^+$, CD4$^+$ lymphocytes, and CD61 PMP percentages expressions, total platelets, leukocytes, and lymphocytes counts; SPSS v. 23 software, IBM, 2015 were used. Data were analyzed by Levene test for homogeneity of variances of samples, while paired T test, ANOVA, were used to establish the differences between samples and controls, and $P < .05$ was considered statistically significant. Pearson correlations were established for AML and BCL subgroups of patients between CD61 PMP and CD3$^+$, CD4$^+$ lymphocytes expressions.

Receiver operating characteristic (ROC) and area under the curve (AUC) made by MedCalc v. 14.8.1, 2014, were used to establish the accuracy of the biomarkers in AML/BCL diagnoses. The sensitivity and specificity of CD-biomarkers are represented by the Youden index which is the optimal cut-off point as the value that maximized the area under the ROC curve.

Figures 1, 2, 6 and 7 were made by the MedCalc program. Figures 3–5 were made with Attune Cytometric Software v.1.2.5, Applied Biosystems, 2010.

3. Results

CD61 expressions (S1-13.30 ± 1.00 vs C1-33.03 ± 3.02, $P < .01$; S2-15.31 ± 0.62 vs C2-56.79 ± 2.70, $P < .01$) and the CD42b percentages (S1-12.83 ± 0.60 vs C1-48.76 ± 0.81, $P < .01$; S2-21.61 ± 0.30 vs C2-49.23 ± 1.22, $P < .01$) were statistically significantly lower in both subgroups of patients with AML compared with the controls (Figs. 1, 3 and 4). T helper/inducer lineage CD4$^+$ (S1-71.32 ± 1.09 vs C1-67.05 ± 0.76, $P < .01$; S2-64.94 ± 0.88 vs C2-59.92 ± 1.33, $P < .01$) and T lymphoid lineage
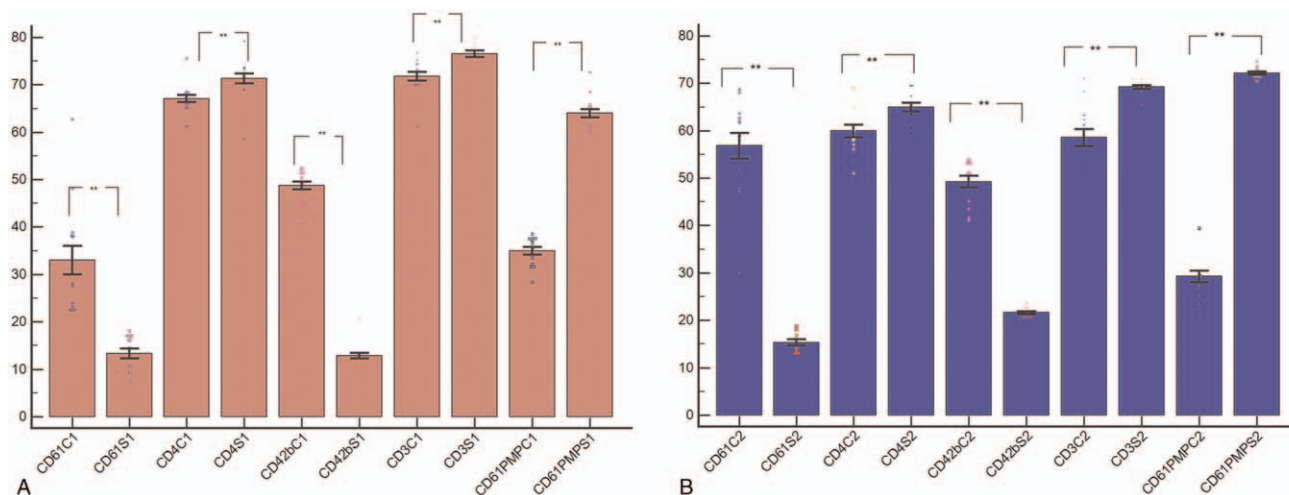
Figure 1. Platelet membrane glycoproteins, T lymphocytes, platelet microparticles-CD61 in patients with acute myeloblastic leukemia reported to controls, in the function of patients' age: (A) ≤65 yrs and (B) >65 yrs. All results were presented as mean values with standard errors. CD61 PMP, platelets derivate microparticles. $^{**}P < .01$ represents significant statistical differences between controls and samples made by paired samples T test.
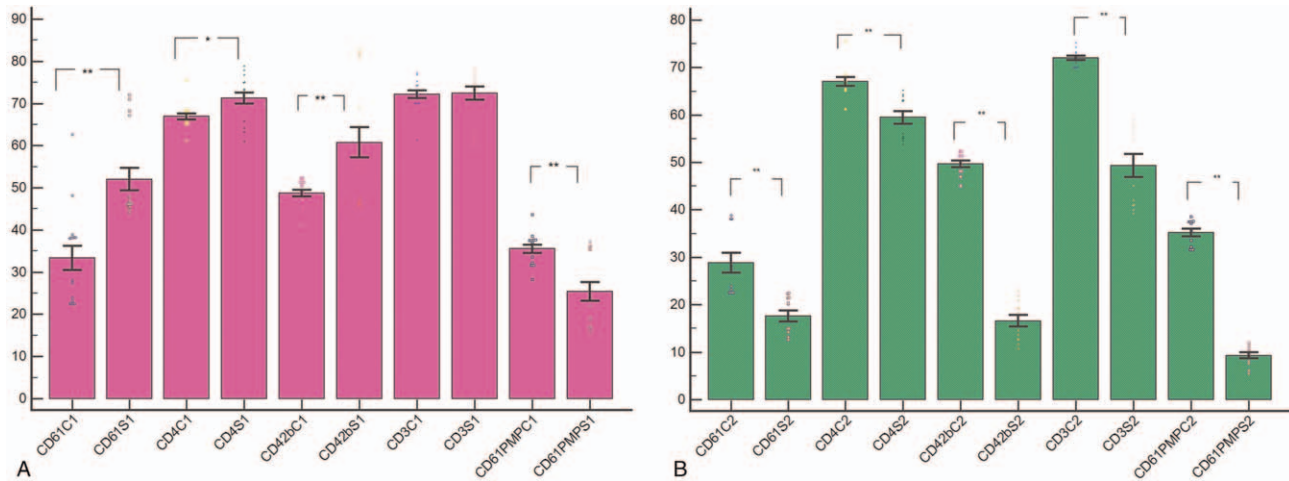
Figure 2. Platelet membrane glycoproteins, T lymphocytes, platelet microparticles-CD61 in patients with B cell lymphoma reported to controls subgroups, in the function of patient's tumoral stages, lymphadenopathies nodes, and splenomegaly: (A) T1–T2 N1–N3 S0–S1; (B) T3–T4 N2–N3 S1–S2. All results were presented as means values with standard errors. T1–T2 N1–N3 S0–S1-Ann Arbor I–II tumoral stages (T1–T2), with lymphadenopathies nodes (N1–N3), and without or with splenomegaly (S0–S1); T3–T4 N2–N3 S1–S2-Ann Arbor III–IV tumoral stages, with lymphadenopathies nodes (N2–N3) and splenomegaly (S1–S2). CD61 PMP, platelets derivate microparticles. $^{**}P < .01$ represents significant statistical differences between controls and samples made by paired samples T test; $^{*}P < .05$ represents significant statistical differences between controls and samples made by paired samples T test.

CD3$^+$ (S1-76.53 ± 0.73 vs C1-71.78 ± 0.91, $P < .01$; S2-69.17 ± 0.35 vs C2-58.53 ± 1.80, $P < .01$) were statistically significantly higher in patients with AML than in controls (Figs. 1, 3 and 4). CD61 PMP had increased values for samples recovered from patients' subgroups with AML in comparison with controls (S1-63.97 ± 0.88 vs C1-34.96 ± 0.82, $P < .01$; S2-72.11 ± 0.32 vs C2-29.26 ± 1.23, $P < .01$, Figs. 1 and 5). The platelets total number presented as associated parameters in Table 1, had lower values with significant statistical differences between AML and controls subgroups of the patients (S1-46,761.00 ± 5749.00 vs C1-238,000.00 ± 13,945.00, $P < .01$; S2-66,159.00 ± 1685.58 vs C2-335,714.28 ± 8939.00, $P < .01$). The leukocytes number from samples of patients with AML (age ≤ 65 years) was statistically significantly higher than in controls (S1-36,303.33 ± 2587.85 vs C1-7018.06 ± 153.93, $P < .01$). Lymphocytes count presented lower values for both subgroups of AML patients in comparison with controls (S1-2683.33 ± 43.00 vs C1-3564.66 ± 152.66, $P < .01$; S2-2152.14 ± 52.70 vs C2-2653.57 ± 135.92, $P < .01$, Table 1).

Because CD61 PMP (S1-64% vs S2-73%) plays an important role in immune response by T regulatory cells activation, these are correlated with estimated percentage of CD3$^+$ lymphocytes (S1-77%, $r = -0.233$, $P > .05$, and S2-72%, $r = 0.451$, $P > .05$) and CD4$^+$ lymphocytes count (S1-72%, $r = -0.621$, $P < .05$ and S2-

65%, $r = -0.530$, $P > .05$, Table 2) in subgroups of AML patients.

First subgroup of patients with low stage BCL presented statistically significant higher values for CD61, CD4$^+$, and CD42b expressions (S1-51.99 ± 2.65 vs C1-33.35 ± 2.84, $P < .01$; S1-71.26 ± 1.32 vs C1-66.92 ± 0.72, $P < .05$; S1-60.74 ± 3.62 vs C1-48.66 ± 0.76, $P < .01$) than controls (Figs. 2–4). CD61 PMP had statistically lower values for samples recovered from patients with low stage BCL reported to controls (S1-25.45 ± 2.20 vs C1-35.50 ± 0.94, $P < .01$, Figs. 2 and 5).

Second subgroup of patients with high stage BCL had significant lower values for all CD-biomarkers reported to controls (CD61-S2-17.61 ± 1.16 vs C2-28.87 ± 2.09, $P < .01$; CD4$^+$-S2-59.45 ± 1.33 vs C2-67.00 ± 0.94, $P < .01$; CD42b-S2-16.60 ± 1.24 vs C2-49.65 ± 0.65, $P < .01$; CD3$^+$-S2-49.29 ± 8.36 vs C2-72.02 ± 1.76, $P < .01$; CD 61 PMP-S2-9.30 ± 0.60 vs C2-35.16 ± 0.81, $P < .01$, Figs. 2–5).

Platelet count in patients with low stage BCL was significantly higher than in controls (S1-290,937.00 ± 16,261.01 vs C1-238,125.00 ± 13,045.39, $P < .05$), while in patients with high stage BCL, platelet count revealed significantly lower values than in controls (S2-180,000.00 ± 13,595.89 vs C2-222,500.00 ± 11,878.48, $P < .05$). Higher values of leukocytes number were observed in both experimental subgroups of patients with BCL

Table 1

Total numbers of platelets, leukocytes, and lymphocytes divided in the function of patient's age with acute myeloblastic leukemia reported to controls.

Parameters	Age ≤ 65 yrs X ± SE		Age > 65 yrs X ± SE	
	Control (C1)	Sample (S1)	Control (C2)	Sample (S2)
Platelets	238,000.00 ± 13,945.00*	46,761.00 ± 5749.00*	335,714.28 ± 8939.00*	66,159.00 ± 1685.58*
Leukocytes	7018.06 ± 153.93*	36,303.33 ± 2587.85*	4912.85 ± 262.38	5328.57 ± 2361.27
Lymphocytes	3564.66 ± 152.66*	2683.33 ± 43.00*	2653.57 ± 135.92*	2152.14 ± 52.70*

SE, standard error; X, obtained results means.
* $P < .01$ represents significant statistical differences between controls and samples made by paired samples T test.

Table 2

Percentage of CD 61 PMP correlated with CD3+, CD4+ lymphocytes expressions at subgroups of patients with acute myeloblastic leukemia.

Pearson correlations (r)	CD 3+		CD 4+	
	Age ≤ 65 yrs	Age > 65 yrs	Age ≤ 65 yrs	Age > 65 yrs
CD 61 PMP	−0.232	0.451	−0.621	−0.530
P	.405	.106	.014*	.857

CD3+, protein complex-specific antigen for T cells; CD4+, glycoprotein found on the surface of the T helper cells; CD61 PMP, platelets derivate microparticles.
* P<.05 represents significant statistical differences between parameters.

reported to controls (S1-14,000.00±730.29 vs C1-7036.93± 145.22, *P* < .01; S2-12,508.33±1362.00 vs C2-7091.75± 101.32, *P* < .01). Lymphocytes number had increased value for patient's subgroup with low stage BCL and decreased value for patient's subgroup with high stage BCL in comparison with control subgroups (S1-3956.25±76.35 vs C1-3604.37±148.02, *P* < .05; S2-3266.66±704.24 vs C2-3547.50±97.53, *P* < .05, Table 3).
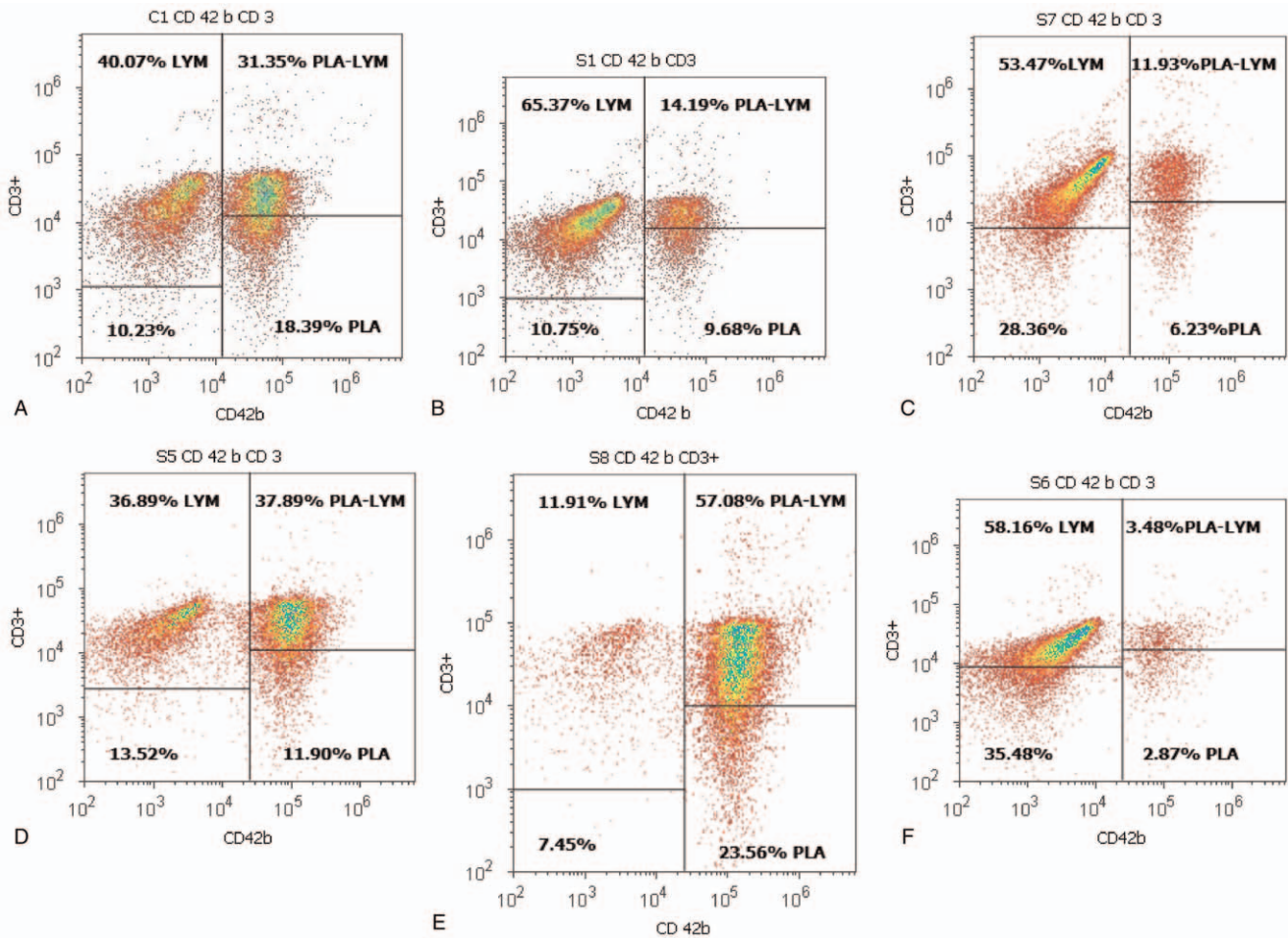
Patients with low stage of BCL present the estimated CD61-PMP percentage (26%) positive correlated with estimated percentages of CD3+, CD4+ lymphocytes (CD3+-73%, *r* =

0.537, *P* < .05, CD4+-72%, *r* = 0.246, *P* > .05). Also were observed correlations between CD3+, CD4+ lymphocytes (CD3+-50%, *r* = 0.822, *P* < .01; CD4+-60%, *r* = −0.798, *P* < .01, Table 4), and CD61 PMP quantity (10%) in patients with high stage BCL.

To establish the diagnostic of laboratory, the predictive model (ROC curves) is used to estimate the risk of adverse outcome based on patients, in medical research.[25] ROC curves were used to show accuracies of positive and negative value predictions (PPV and PNV, Table 5) in relationship with TPR (sensitivity) and FPR (1-specificity) for each CD-biomarker. A single test
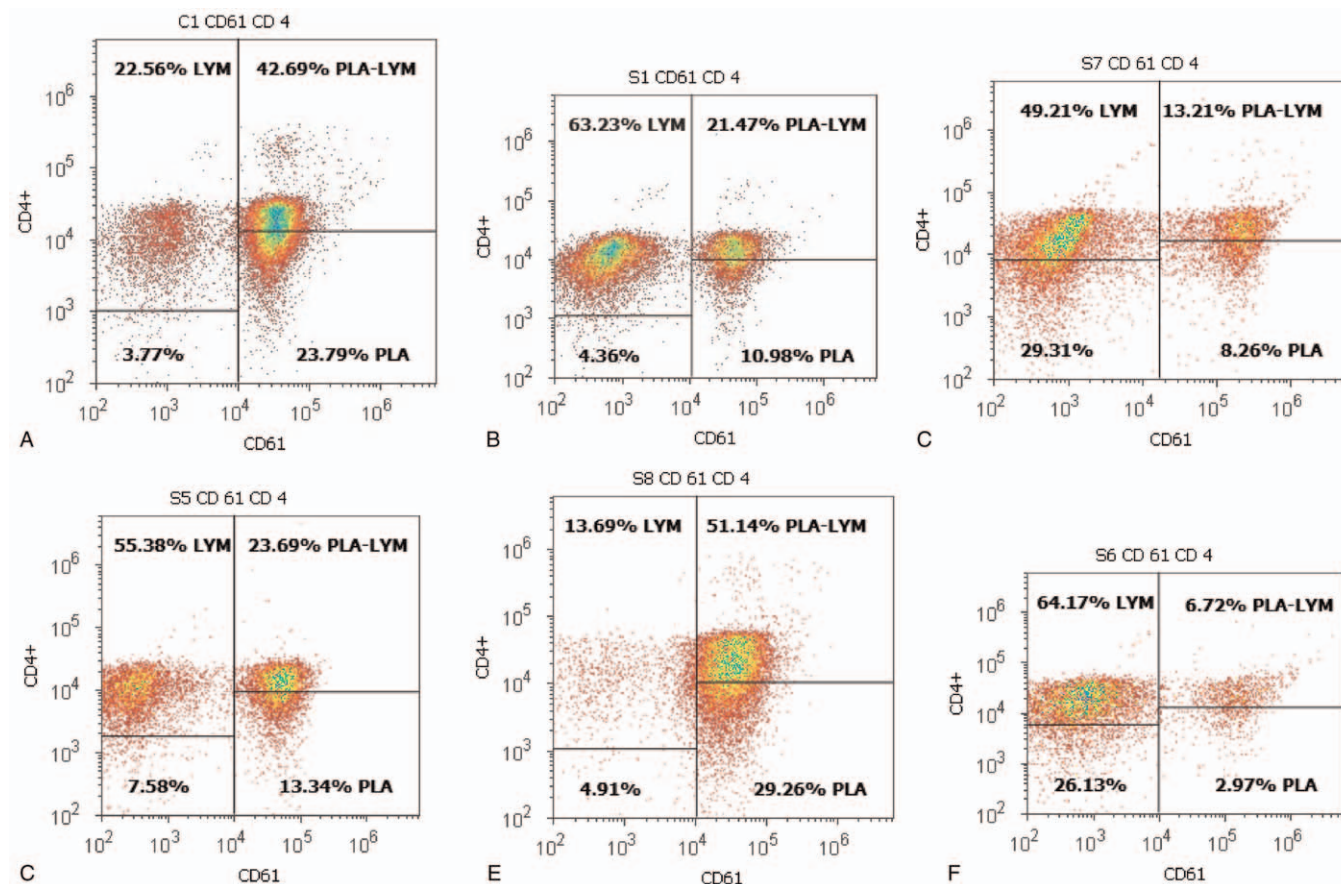
Figure 3. Expressions of the CD61/CD4+ by flow cytometry: (A) healthy patients; (B) patients with AML; (C) BCL patients with Ann Arbor I tumoral stage of BCL with axillary lymphadenopathies nodes (N1); (D) BCL patients with Ann Arbor II tumoral stage with axillary and laterocervical lymphadenopathies nodes (N2); (E) BCL patients with Ann Arbor II tumoral stage with laterocervical, supraclavicular, axillary lymphadenopathies nodes (N3), and splenomegaly (SI); (F) BCL patients with Ann Arbor IV tumoral stage with supraclavicular, axillary, abdominal lymphadenopathies nodes (N3), and splenomegaly (SII). AML, acute myeloblastic leukemia; BCL-B, cell lymphoma; LYM-T, lymphocytes; PLA, platelets; PLA-LYM, platelet–lymphocyte interactions.

Figure 4. Expressions of the CD42b/CD3$^+$ by flow cytometry: (A) healthy patients; (B) patients with AML; (C) BCL patients with Ann Arbor I tumoral stage of BCL with axillary lymphadenopathies nodes (N1); (D) BCL patients with Ann Arbor II tumoral stage with axillary and laterocervical lymphadenopathies nodes (N2); (E) BCL patients with Ann Arbor II tumoral stage with laterocervical, supraclavicular, axillary lymphadenopathies nodes (N3), and splenomegaly (SI); (F) BCL patients with Ann Arbor IV tumoral stage with supraclavicular, axillary, abdominal lymphadenopathies nodes (N3), and splenomegaly (SII). AML, acute myeloblastic leukemia; BCL, B cell lymphoma; LYM-T, lymphocytes; PLA, platelets; PLA-LYM, platelet–lymphocyte interactions.

Figure 5. Platelet-derived microparticles-CD61 expressions by flow cytometry in patients with acute myeloblastic leukemia (B) and low and high stages B cell lymphoma (C and D) reported to control (A) C1 CD61 PMP-platelets derivate microparticles in controls; C1 IgG PMP-negative control (IgG) applied to control platelets derivate microparticles; S1 CD 61 PMP-platelets derivate microparticles from samples recovered from patients with acute myeloblastic leukemia; S1 IgG PMP-negative control (IgG) applied to obtained samples from patients with acute myeloblastic leukemia; S4 CD 61 PMP-platelets derivate microparticles from samples recovered from patients with low stage B cell lymphoma; S4 IgG PMP-negative control (IgG) applied to obtained samples from patients with low stage B cell lymphoma; S6 CD 61 PMP-platelets derivate microparticles from samples recovered from patients with high stage B cell lymphoma; S6 IgG PMP-negative control (IgG) applied to obtained samples from patients with high stage B cell lymphoma.

Table 3

Platelet membrane glycoproteins, T lymphocytes, platelet microparticles-CD61, total numbers of platelets, leukocytes, and lymphocytes in correlations with the clinicopathological aspects of B cell lymphoma patient's subgroups.

Parameters	X±SE I–II Ann Arbor		X±SE III–IV Ann Arbor	
	Control (C1)	Sample (S1)	Control (C2)	Sample (S2)
Platelets	238,125.00±13,045.39*	290,937.00±16,261.01*	222,500.00±11,878.48*	180,000.00±13,595.89*
Leukocytes	7036.93±145.22†	14,000.00±730.29†	7091.75±101.32†	12,508.33±1362.00†
Lymphocytes	3604.37±148.02*	3956.25±76.35*	3547.50±97.53*	3266.66±704.24*

SE, standard error; X, obtained results means.

* $P < .05$ represents statistical differences between controls and samples made by paired samples T test.

† $P < .01$ represents significant statistical differences between controls and samples made by paired samples T test.

Table 4

Percentage of CD 61 PMP correlated with CD3+, CD4+ lymphocytes expressions in subgroups of patients with B cell lymphoma.

Pearson correlations (r)	CD 3+		CD 4+	
	I–II Ann Arbor	III–IV Ann Arbor	I–II Ann Arbor	III–IV Ann Arbor
CD 61 PMP	0.537	0.822	0.246	−0.798
P	.032*	.001†	.359	.002†

CD3+, protein complex-specific antigen for T cells; CD4+, glycoprotein found on the surface of the T helper cells; CD61 PMP, platelets derivate microparticles.

* $P < .05$ represents significant statistical differences between parameters.

† $P < .01$ represents significant statistical differences between parameters.

ROC curve was made for each CD-biomarker with 2 overlapping distributions (control and experimental, Figs. 6 and 7).

True positive values (sensitivity) in patients with AML were increased for CD61 (93.33%), CD4+ (61.29%), CD42b (96.43%), CD3+ (97.07%), and CD61 PMP (96.67%). Specificity (TNV-true negative values) was increased for CD61 (96.43%), CD4+ (96.30%), CD42b (93.33%), CD3+ (68.33%), and CD61 PMP (92.87%, Table 5 and Fig. 6). Sensitivity and specificity of the methods were increased, for patients with BCL: 77.78% and 100.00% of CD61, 60.87% and 100.00% of CD4+, 76.19% and 85.71% of CD42b, 65.79% and 100.00% of CD3+, 75.86% and 100.00% of CD61 PMP (Table 5 and Fig. 7).

4. Discussion

Antigen-specific T cells (CD4+ and CD3+ expressions) play an important role in immune protection toward cancer and represent the cellular basis for specific immunotherapy. Platelets are involved in the hemostasis regulation and interact with leukocytes to avoid substantial blood loss from the circulation.[26] Activated platelets change their shape and size, release cytoplasmic granules contents, and change their glycoproteins expressions. Signals of platelet activation led to biochemical modifications, accompanied by surface glycoproteins changes expressions, which are used in platelet state evaluation.[27–29]

Because flow cytometry is an optimal laboratory method to analyze the platelet function, in this study, we quantify the levels of platelet membrane glycoproteins (CD61 and CD42b) in samples recovered from patients with AML or BCL.

In patients with AML, the means of molecules' expression on platelets' surfaces (CD61 and CD42b) were significant lower than in both control subgroups. Decreased expressions of CD61, were observed in myelodysplastic syndromes (MDS) patients,[30] and the reduced amounts of CD42b were reported to be a common finding in MDS patients.[31]

CD61 and CD42b presence on platelet membranes, as functionally active surface receptors, are factors that mediate the adhesion of active platelets to leukocytes, endothelial cells,

Table 5

Receiver operating characteristic (ROC) analysis of CD-biomarkers in patients with hematological malignancies.

Biomarkers	AUC	95% CI	P	Youden J index	Cut-off value	Sensitivity	Specificity	PPV	PNV
CD61_AML	0.973	0.938–1.000	<.0001*	0.897	≤18.85	93.33	96.43	96.60	93.10
CD61_BCL	0.817	0.697–0.936	<0001*	0.777	>28.14	77.78	100.00	100.00	71.14
CD4+_AML	0.817	0.709–0.926	<.0001*	0.575	>68.50	61.29	96.30	95.00	68.40
CD4+_BCL	0.786	0.651–0.921	<.0001*	0.608	>68.50	60.87	100.00	100.00	78.60
CD42b_AML	0.960	0.894–1.00	<.0001*	0.821	≤23.70	96.43	93.33	93.10	96.60
CD42b_BCL	0.741	0.611–0.872	<.0001*	0.619	≤49.36	76.19	85.71	94.10	54.50
CD3+_AML	0.792	0.671–0.914	<.0001*	0.553	>68.30	97.07	58.33	76.70	93.30
CD3+_BCL	0.733	0.599–0.868	<.0001*	0.657	≤70.12	65.79	100.00	100.00	58.10
CD61 PMP_AML	0.895	0.889–1.000	<.0001*	0.895	>38.54	96.67	92.87	93.50	96.30
CD61 PMP_BCL	0.897	0.817–0.978	<.0001*	0.758	≤24.01	75.86	100.00	100.00	79.40

AUC, area under the curve; AML, acute myeloblastic leukemia; BCL, patients with B cell lymphoma; CD3+, protein complex-specific antigen for T cells; CD4+, glycoprotein found on the surface of the T helper cells; CD42b, CD61, platelet membrane glycoproteins; CD61 PMP, platelets derivate microparticles; CI, confidence interval; PNV, negative value predictions; PPV, positive value predictions; Youden J = sensitivity + specificity − 100.

* Obtained results were significant and statistically different ($P < .0001$).
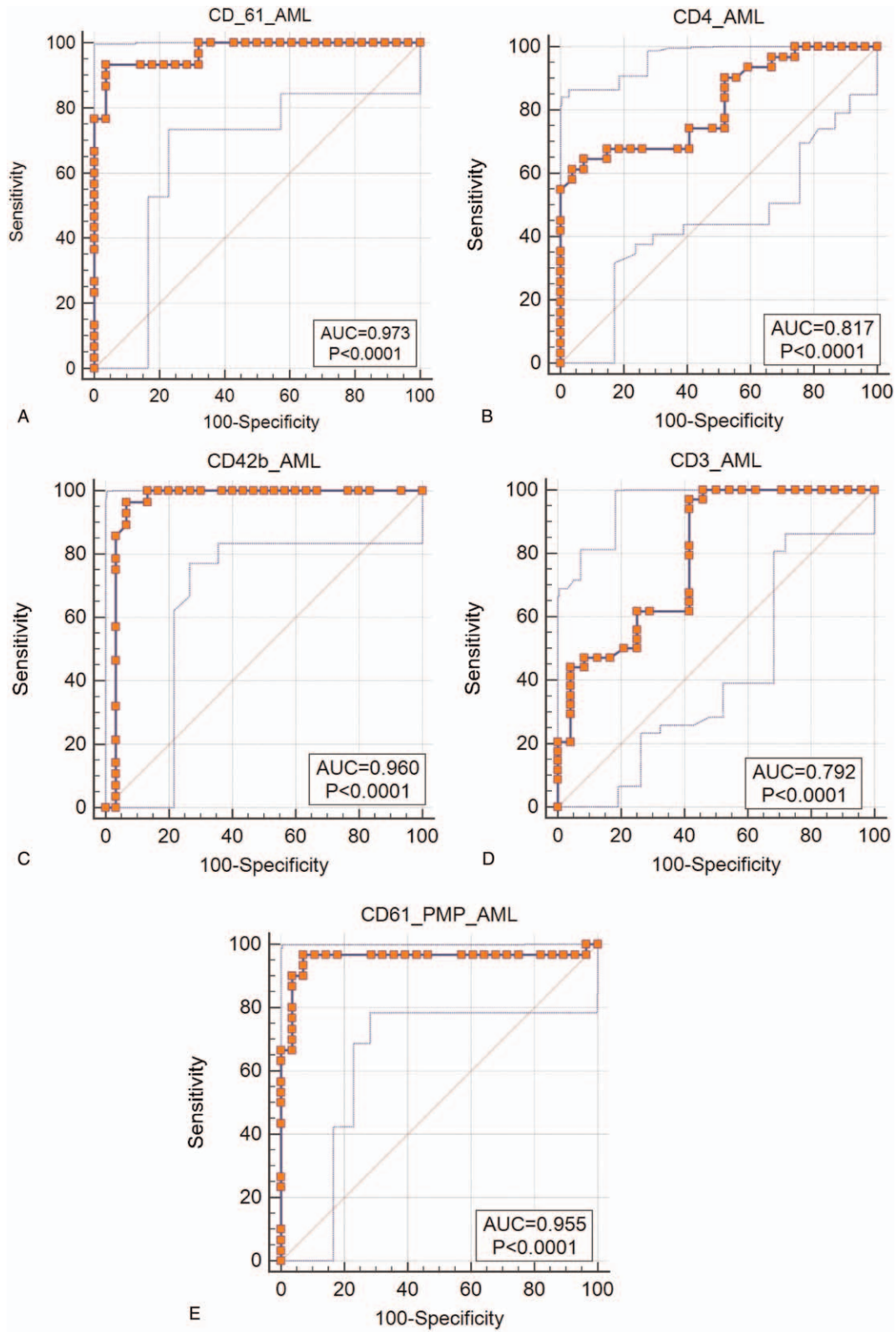
Figure 6. CD-expressions of platelets and lymphocytes, and CD61 platelets derivate microparticles as biomarkers in patients with acute myeloblastic leukemia: (A) platelet CD61 (AUC-0.973); (B) lymphocytes CD4^{+} (AUC-0.817); (C) platelet CD42b (AUC-0.960); (D) lymphocytes CD3^{+} (AUC-0.792); (E) CD61 PMP (AUC-0.955).
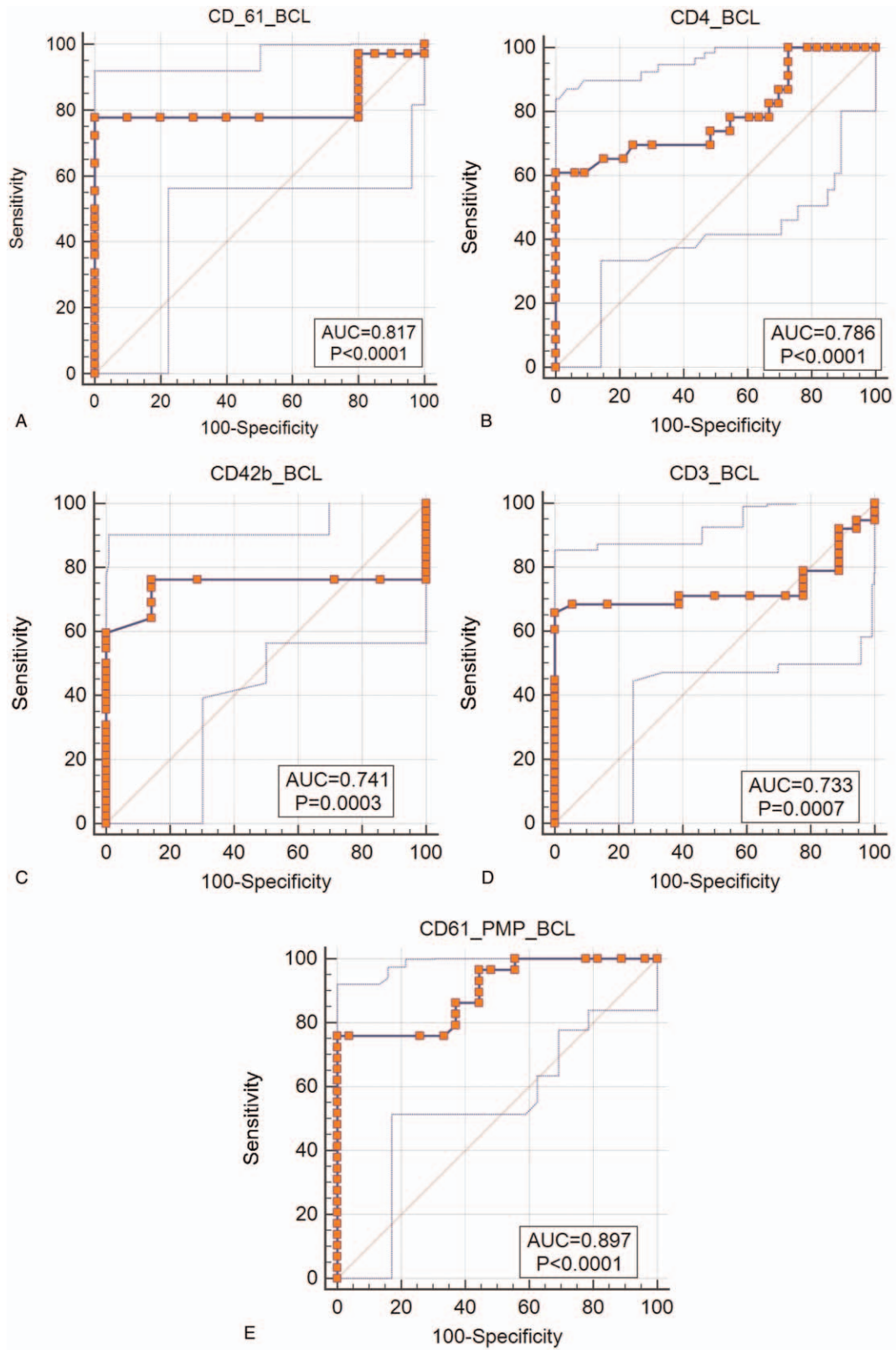
Figure 7. CD-expressions of platelets and lymphocytes, and CD61 platelets derivate microparticles as biomarkers in patients with B cell lymphoma: (A) platelet CD61 (AUC-0.817); (B) lymphocytes CD4+ (AUC-0.786); (C) platelet CD42b (AUC-0.741); (D) lymphocytes CD3+ (AUC-0.733); (E) CD61 PMP (AUC-0.897).

and cancer cells. Platelet activation in patients with low stage BCL manifests itself by increased expressions of CD61 and CD42b antigens, and in patients with AML manifests itself by increased expressions of microparticles derivate platelet-CD61. Increased levels of CD61, CD42b, and CD61 PMP, both in resting state and in in vivo active platelets, intensify the ability of platelets to adhesion and aggregation, increases the risk of thromboembolic events, promotes tumor cells proliferation, angiogenesis, and disease progression, in conformity with scientific literature.[27,29–32]

PMPs have an 18% to 28% contribution to both procoagulant and anticoagulant activities of human platelets stimulated with different platelet agonists. The percentage was higher in unstimulated platelets (28–40%). Leukemia cells can damage vascular endothelial cells, they interconnect with platelets and stimulate platelet activation.[33]

Modified levels of plasma PMPs were associated with bleeding or thromboembolic complications. In normal conditions, the presence of PMPs inhibits coagulation rather than promotes coagulation,[34] but the increased PMPs presence in clinical conditions may be a biomarker of ongoing thrombosis.[35] Increased PMPs count was observed in the blood of patients with thrombotic and inflammatory disorders.[36–39] The relationship between persistent immune system activation and coagulation/inflammation is poorly understood. Interactions between platelets and monocytes are well described by the scientific literature, but little is known about the interaction between platelets and the adaptive immune system. Platelet–T cell complex plays a role in the fast recruitment of antigen-experienced T cells to the inflammation/coagulation place. This mechanism maintains the procoagulation/inflammation status in patients, contributing to the disease pathology.[40]

An increased number of platelets in circulation and their ability to release pro-inflammatory and anti-inflammatory mediators, which are stored in secretory granules, suggest that platelets are critical players in the early phase of the host immune response.[41,42] Platelets are known to be involved in functions beyond hemostasis and were identified as regulators of both innate and adaptive immune systems.[43,44]

Our results agree with the scientific literature presented above because T helper/inducer lineage CD4+ and T lymphoid lineage CD3+ expressions presented significant differences between patients with AML, with low stage BCL, and those in matching control subgroups. In our study activation of the platelets was revealed by a significant increase of CD61 PMP in patients with AML and by increased expressions of CD61 and CD42b in patients with low stage BCL in comparison with control subgroups of patients. Furthermore, total numbers of platelets and leukocytes were significantly higher in patients with low stages BCL compared to controls. In contrast, in patients with high stage BCL, mean values of CD4+ and CD 3+ levels, CD 61, CD42b expressions, CD61 PMP, the total number of platelets, and leukocytes count were significantly decreased reported to control subgroups of patients.

In recent years, increasing evidence supports the notion that platelets participate in immune responses and interactions between platelets and leukocytes contribute to both thrombosis and inflammation.[45,46] Platelet–leukocyte complexes were observed in peripheral blood in patients with myeloproliferative disease.[47]

Because the tumor environment is one of inflammation, the tumor cells release chemokines and cytokines that attract inflammatory cells, which in turn release factors that the tumor cell uses to survive, proliferate, and metastasize.[48] Platelets were implicated in this process, and the platelet count is inversely correlated with survival.[49] Platelets and leukocytes can form complexes with tumor cells that facilitate tumor cell attachment to vessels and invasion of the tissue. P-selectin on both endothelial cells and platelets and the α4β1 integrin on myeloid cells [50] are implicated in metastasis. Platelets and leukocytes release factors that destabilize existing vessels, promote capillary sprout formation, and enhance endothelial cell proliferation.[51]

In this study, because CD61 PMP plays an important role in the immune system by T regulatory cell activation, these are negatively correlated with CD4+ lymphocytes in the subgroup of AML patients (age ≤ 65 years). Also, CD61-PMP percentage was presented a positive correlation with the percentage of CD3+ lymphocytes for both subgroups of patients with BCL, and a negative correlation of this with CD4+ lymphocytes in the high stage of BCL patients.

Results of some clinical studies have shown that the leukocytes–platelet interactions are directly correlated with platelet and leukocyte counts, and with the increased expression of leukocyte biomarkers.[52,53] Role of platelet microparticles in cancer development is unknown, but it is well established that tumor cells can activate platelets and induce platelet aggregation. The authors demonstrated that platelet microparticles levels are strongly correlated with aggressive tumors and a poor clinical outcome.[54]

High levels of the T regulatory cells in the tumor microenvironment are associated with poor prognosis in many cancers, such as ovarian, breast, colorectal, ovarian, renal, and pancreatic cancer.[55–60] In some types of cancer the opposite is true, and high levels of T regulatory cells are associated with a positive prognosis, in cancers such as colorectal carcinoma and follicular lymphoma. T regulatory cells can trigger cell proliferation and metastasis and this opposite effect indicate that the T regulatory role in the development of cancer is dependent on both the type and location of the tumor.[56]

Increased levels of CD61 PMP in AML patients and CD61 and CD42 B expressions in BCL patients indicate platelet activation. Since we found that platelet activation was associated with platelet binding to lymphocytes in AML and BCL patients, it is likely that platelet binding modifies the lymphocyte function. Authors showed that platelet bound preferably to memory T lymphocytes.[61,62]

In this study, we observed the accuracy of the potential biomarkers for AML and BCL patients. As expected, flow cytometry represents the most sensitive modality for immunophenotyping. Methods sensitivity average was $80.12 \pm 14.79\%$ and the specificity average of the methods was $92.29 \pm 12.77\%$. Predictive positive values average (94.90 ± 7.02) and predictive negative values average (78.94 ± 15.71) means that the flow cytometry analysis methods had good accuracies to obtain the results. The average of the area under the curve presented by ROC curves (AUC $\pm$ SE) was 0.841 ± 0.085 which means good statistical positive and negative results distribution for both groups of patients and controls. Our obtained results conform with recent studies about the diagnosis of acute leukemia and Non-Hodgkin lymphomas methods.[63–67]

In this study, limitations to develop the utility of these observed biomarkers were the fact that were analyzed only for AML and BCL from all diversity of hematological malignancies, and a small number of samples recovered from treated patients to be able to

study the subtypes of AML and NHL. However, we cannot eliminate that medication of AML and BCL patients could affect lymphocytes–platelets interactions. Because this represents a limitation, a study of lymphocyte–platelet complexes in a large cohort of patients before and after taking drugs will be required to study the modulation of these. We show in this study that platelets can bind to lymphocytes in AML and BCL patients, and this interaction is associated with altered immune response. Another limitation is that lymphocyte–platelet complexes were analyzed at a one-time point in each patient during the treatment. Further studies analyzing the same patient before and after taking drugs will be required to validate the potential of lymphocyte–platelet complexes in immune response in patients with AML and BCL. Our observations suggest that controlling these interactions is beneficial for the therapeutic regulation of the patient's immunity.

5. Conclusions

Platelet–leukocyte interactions are involved in tumor diseases, which supports that platelets represent an immune system fundamental part. During these interactions, platelets trigger intracellular signaling in immune cells and they modulate the immune responses.

Platelet activation in patients with low stage B cell lymphoma manifests itself by increased expressions of CD61 and CD42b antigens, and in patients with acute myeloblastic leukemia manifests itself by increased expressions of microparticles derivate platelet-CD 61.

Future directions in this research area will be to study a variety of hematological malignancies subtypes and to establish the valid correlations between observed biomarkers and DNA content and cell apoptosis before and after taking drugs at patients.

Because of the importance of platelet activity in many clinical conditions, some assay of platelet activation will eventually be widely utilized. Another direction to study in correlations with our biomarkers is the DNA content because flow cytometers provide a rapid method to measure altered DNA content, ploidy, and analysis of cell division kinetics (S-phase fraction, mitotic index) has important implications in areas such as clinical tumor prognosis. Also, other future directions correlated with our biomarkers will be the analysis of cell apoptosis, because it is a major research application of flow cytometry and may become a new clinical application for determining the efficacy of chemotherapy in patients with different types of malignancies. Also, our findings suggest that the determination of the levels of circulating lymphocytes with bound platelets in AML and BCL patients may prove to be a useful tool to follow up the activities of the diseases. On the other hand, understanding how platelet microparticles impact different diseases will enable the identification of new cellular pathways that are amenable to therapeutic manipulation. Besides, platelet microparticles show promise as a diagnostic biomarker for diseases and potentially as a delivery system for therapeutics.

Acknowledgments

Author contributions

All authors contributed equally to this study and share the first authorship. EM, MA, and MMG designed the research; EM and MM performed the experiments; EM performed the statistical analysis, made the tables and the figures; EM is a certified specialist in biostatistics and computational uncertainty measurement and performance indicators in the laboratory; LP is an expert biostatistician; EM, AM, AC, and ND wrote the manuscript; EM and EG revised and corrected the manuscript.

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