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Clinical Significance of Flow Cytometry Findings in Brazilian Patients with De Novo Acute Myeloid Leukemia

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Abstract

Introduction: Immunophenotyping by Flow Cytometry (FC) is an essential method for diagnosis and classification of Acute Myeloid Leukemias (AML), and its extensive use could identify blast cell subpopulations with phenotypes rarely seen in normal myelopoiesis, correlating with clinical, morphological and prognostic characteristics.

Methods: In this study we analyzed 143 cases of AML, examining them for Leukemia-Associated Immunophenotype (LAIP) by FC immunophenotyping in leukemic cells using a panel of monoclonal antibodies (MoAb) for diagnosis and classification of Acute Leukemia (AL). At the same time, clinical, demographic and hematological data of these patients were also investigated. Most patients were male adults and splenomegaly and hepatomegaly were present in most cases.

Results: Immunophenotyping showed a characteristic profile of AML with expression of pan-myeloid antigens CD13, CD33 and Myeloperoxidase (MPO), combined with CD34 and CD117 in most cases. Expression of CD14 and CD64 were observed in AML with monocytic component (AML-M4/M5), CD235a, CD36 and CD71 in cases of erythroleukemia (AML-M6) and platelet glycoproteins CD41, CD42b and CD61 in acute megakaryocytic leukemia (AML-M7). Regarding the aberrant phenotype, higher levels of expression of CD4, CD7 and CD56 were observed, corresponding to 24.5%, 22.4%, and 16.1% of the cases, respectively.

Conclusion: We conclude that LAIP, as they are described here, were present in the vast majority of cases of investigated AML, with a relevant association with prognostic factors, clinical data, cytomorphological classification.

Keywords: Immunophenotyping, Flow cytometry, Monoclonal antibodies, Acute myeloid leukemia, Leukemia-associated immunophenotype

Introduction

The characterization of Acute Leukemias (AL) is based on multiparametric analysis which includes clinical features, cell morphology, genetics and immunological markers [1,2]. These parameters have been shown to be important for the diagnosis and prognosis [1]. The first systems of classification of AL were based on cytomorphological investigations enabling the differentiation between Acute Myeloid Leukemia (AML) and Acute Lymphoid Leukemia (ALL) [1-4].

The system established by the French-American British group (FAB), established a morphological classification as AML

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according to the characteristics of the cells obtained from the Bone Marrow (BM) aspirate in: AML-M0 (Undifferentiated AML); AML-M1 (AML with minimal differentiation); AML-M2 (Maturing AML); AML-M3 (Acute promyelocytic leukemia or APL); AML-M4 (Acute myelomonocytic leukemia), including the eosinophilic form (AML-M4eos); AML-M5 (Acute monocytic leukemia), subdivided into AML-M5a with predominance of monoblasts and AML-M5b with predominance of promonocytes; AML-M6 (Erythroleukemia); AML-M7 (Acute megakaryoblastic leukemia) [3,4].

Currently, cytomorphology remains a central tool in the diagnostic and classification of hematological disorders [2]. It should be integrated with other methods such as flow cytometry, cytogenetics/molecular genetics and clinical data [1,2].

Flow Cytometry (FC) is widely used for diagnosis and monitoring of hematological neoplasm, being a quicker and simpler technique than other methods. Its high sensitivity and antigenic quantification enables the identification of cells with Leukemia-Associated Immunophenotype (LAIP) in the diagnosis and detection of Minimal Residual Disease (MRD) through a panel of Monoclonal Antibodies (MoAb) conjugated with fluorochromes that recognize specific cell antigen epitopes, allowing a more accurate delineation of the LAIP, enabling the differentiation between AML and ALL [5-11].

Thus, the systematic use of MoAbs, such as CD13, CD33, CD65, c-Kit receptor (CD117), antigen associated with hematopoietic precursors (CD34), HLADR and anti-myeloperoxidase (MPO), allow the definition of AML, being essential for the definition of subtypes M0, M7, M5a and variant hypogranular form of APL (AML-M3v) [10-15].

In addition, to contributing to the diagnosis, FC immunophenotyping can also evidence blast cell heterogeneity as reflected by the existence of a high variety of phenotypes as well as detect antigen associations rarely seen in normal BM cells, such as aberrant expression of lymphoid antigens and asynchronous phenotypes [13].

To characterize the other FAB subtypes of AML, FC is less important, but corroborates with the cytomorphological findings in diagnosis and differentiation between the M1 and M2 subtypes [10-13]. The expression of CD14 and CD64, are present in the monocyte population, characterizing AML-M4/M5 and erythroleukemia by the expression of glycophorin alpha (CD235a) [6-8,13-15].

In order to assess the occurrence of LAIPs and correlate their presence with the various morphological subtypes, we analyzed 143 de novo AML cases. Correlations between clinical data, aberrant phenotypes, patient age and hematologic changes were also examined.

Methods

Study Population

A total of 143 newly diagnosed AML cases at the Blood Center Dalton Cunha (HEMONORTE), Natal City, located in Northeastern Brazil in the period 2016-2018 were included in the study. Ethical

approval for this study was granted by the Ethics and Research Committees of the Onofre Lopes University Hospital of the Federal University of Rio Grande do Norte (CAAE: 51300215.6.0000.5292). All patients or their legal representatives signed the informed consent form.

Demographic and clinical data for each case were obtained at the time of diagnosis, including age, sex, fever, splenomegaly, hepatomegaly, adenopathy, hemorrhagic phenomena and presence of tumor masses. Hematologic information collected, such as White Cell Count (WCC), platelets, hemoglobin and B12 vitamin measurement. In Peripheral Blood (PB). And BM smears examination supplemented by cytochemical of Sudan Black B (SBB) and MPO stained and FC immunophenotyping were performed to confirm AML [16-20].

Hematological analysis from peripheral blood and bone marrow

Hematologic information was performed in a hematological analyzer (BC-3000 Plus, Myndray, China). Differential blood cell counts were performed in PB smears stained with May-Grünwald-Giemsa (MGG-Laboclin, Brazil), in which a minimum 100 mononuclear cells were counted in an optical microscope using 20x and 100x objective lenses (Zeiss Microscope, Götting, Germany) and the result of the cell count scored in percentage. MGG-staining smears of BM were evaluated according to the FAB criteria [3,4].

Serum dosage of vitamin B12 was performed by chemiluminescence immunoassay (ARCHITECT i1000SR immunoassay analyzer, Abbott, USA) with the aim of excluding Megaloblastic Anemia (MA) in patients with suspected AML-M6.

Immunophenotyping studies

Erythrocyte-lysed whole BM samples were analyzed by FC using a large panel of MoAbs in four combinations (**Table 1**) [17-20]. Detection of surface, cytoplasmic (cyt) such as MPO, cytCD13, cytCD79a, cytCD22, cytCD3, anti-IgM and nuclear (nu) Terminal Deoxynucleotidyl Transferase (TdT) antigens were performed following a previously established protocol [19-23].

Data acquisition and analysis were performed on a FACScalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San José, CA, USA) using Cell-Quest software. Calibration and fluorescence compensation were carried out using Calibrite beads (Becton Dickinson, San José, CA, USA) and immunoglobulin isotype-matched negative controls. Identification of blast cells was performed using Forward Scatter (FSC) versus Side Scatter (SSC) parameters and/or CD45 intensity versus SSC dot plots. Antigen expression was considered to be positive when the percentage of positive blast cells was equal or greater than 20% [19,20].

Results

Out of the 143 cases, 85 males and 58 females were studied. Patients were aged between 02 and 92 years. The age group most affected by the disease was 20 to 59 years (65 cases), followed by individuals over the age of 60 with 52 patients. All cases were classified according to FAB criteria and FC immunophenotyping

Table 1: Monoclonal antibodies used in this study.

MoAb/Fluorochromes	Clone	Reactivity	Source
CD45 _{APC}	HI3	Common leukocyte antigen	BD
HLADR _{APC}	L243	MHC class II cell surface receptor	BD
CD34 _{PerCP}	8G12	Immature hematopoietic precursors	BD
CD117 _{APC}	CE/IVD	c-kit receptor / Myeloid precursor	BD
anti-MPO _{FICT}	CLB	Myeloperoxidase / Myeloid antigen	BD
CD13 _{FICT}	L13	Aminopeptidase N / Myeloid antigen	BD
CD33 _{PE}	P67.6	Myelomonocytic antigen / Myeloid precursors	BD
CD36 _{PE}	NLO7	Scavage receptor	BD
CD14 _{PerCP}	M5E2	Lipopolysaccharide receptor / monocytes antigen	BD
CD64 _{APC}	MB22	Precursor monocytes antigen	BD
CD65 _{FICT}	88H	Myeloid antigen / Myeloid precursors	BD
CD66b _{FICT}	G10F5	Carcinoembryonic antigen / myeloid antigen	BD
CD71 _{FICT}	L01.1	Transferrin receptor	BD
CD235a _{FICT}	GA-R2	Glycophorin A / eritroid antigen	BD
CD41 _{FICT}	HIP8	Glicoprotein IIb/ Platelet and platelet precursors	BD
CD42b _{FICT}	HIP1	Glicoprotein IX / Platelet and platelet precursors	BD
CD61 _{PerCP}	RUU-PLF12	Glicoprotein IIIa / Platelet and platelet precursors	BD
CD1a _{PerCP}	HI-149	Thymocytes antigens / T-lymphocytes precursors	BD
CD2 _{FICT}	S5.2	T-lymphocytes mature and precursors	BD
CD3 _{APC}	UCHT1	Best marker for cells of T lineage	BD
CD7 _{APC}	M-T701	T-cell lineage-associated antigen	BD
CD56 _{PE}	B159	Natural Killer Cells	BD
CD10 _{FICT}	HI10	CALLA antigen / B-lymphocytes precursors	BD
CD19 _{PerCP}	89B	Pan-B lineage lymphocytes	BD
CD20 _{FICT}	2H7	Pan-B lineage lymphocytes	BD
CD22 _{FICT}	HIB22	Pan-B lineage lymphocytes	BD
CD79a _{PE}	HM47	Early B-lymphocytes precursors	BD
IgM _{APC}	G20-123	IgM immunoglobulin heavy chain	BD
TdT _{PE}	HT9	Terminal-deoxynucleotidyl Transferase	BD
Multitest conjugated MoAb			
BD/Simultest Leucogate: CD14FICT/CD45PE: CD14 mature monocytes and CD45 common leukocyte antigen;			
BD/Oncomark CD14FICT/CD64PE: Monocytic development;			
BD/Oncomark CD7FICT/CD33PE: CD7 T-lymphocytes and CD33 myelomonocytic antigen; BD/Oncomark /CD15FICT/CD34PE: CD15 mature granulocytes and CD34 Immature precursors; BD/Multitest 4-Color: CD3FICT/CD8PE/CD45PerCP/CD4AP: T-subsets lymphocytes;			
BD/Multitest 4-Color: CD3FICT/CD16-56PE/CD45PerCP/CD19AP: T, NK and B lymphocytes			
IgG1FITC/IgG1PE/IgG1PerCP/IgG1APC: BD Isotypic matched antibodies			
Note: Monoclonal antibodies (MoAb); Fluorescein isothiocyanate (FITC), Phycoerythrin (PE), Peridinin-chlorophyll protein (PerCP), Allophycocyanin (APC), Becton & Dickinson (BD).			

distributed into: 03 AML-M0, 53 AML-M1, 22 AML-M2, 09 AML-M3, 27 AML-M4, 14, AML-M5a, 08 AML-M5b, 04 AML-M6 and 3 AML-M7 (Table 2).

Clinical data associated with the AML were present in most cases. Of these, splenomegaly was predominant with 118 cases, followed by hepatomegaly and bone pain with 99 and 40 cases respectively. Hemorrhagic phenomena were predominantly in APL and directly associated with low platelet count in PB. Presence of gingival hypertrophy and tumor mass were predominant in AML-M5a (Table 2).

A WBC variation was observed, which ranged from 700 to 299.000/ μ L. There was a predominance of patients with WBC more than 50.000/ μ L with 82 cases, followed by 40 cases with WBC between more than 10.000 and 50.000/ μ L. Patients with

WBC between 5.000 and 10.000/ μ L and less than 5.000/ μ L were observed in 11 and 10 cases, respectively. Hemoglobin levels less than 12.0g/dL and thrombocytopenia were observed in most cases, with more pronounced results in cases of APL. Cytochemical stain of SBB and MPO were positive in most cases, notably in subgroups M1, M2 and M3, and negative in AML-M0, M6 and M7 (Table 3).

In FC analysis, leukemic cells were initially identified by FSC/SSC ratio and expression of pan-myeloid antigens: CD13, CD33, CD65, CD117 and MPO in most cases. CD34 was expressed in most cases, characterizing the presence of blast cells with little differentiation (Table 4 and Figure1).

Leukemic cells from APL showed a characteristic pattern of positivity to CD13, CD33, MPO and CD117 and negativity for CD34

Table 2: Correlation between demographic and clinical date and FAB subgroups M0 into M7 of patients with acute myeloid leukemia.

Date	Patients n=143	AML-M0 n= 03 n+ (%)	AML-M1 n= 53 n+ (%)	AML-M2 n= 22 n+ (%)	AML-M3 n= 09 n+ (%)	AML-M4 n= 27 n+ (%)	AML-M5a n= 14 n+ (%)	AML-M5b n= 08 n+ (%)	AML-M6 n= 04 n+ (%)	AML-M7 n= 03 n+ (%)
Gender										
Male	85	02 (66.7)	31 (58.5)	13 (59.1)	07 (77.8)	17 (63.0)	07 (50.0)	06 (75.0)	01 (25.0)	01 (33.3)
Female	58	01 (33.3)	22 (41.5)	09 (40.9)	02 (22.2)	10 (37.0)	07 (50.0)	02 (25.0)	03 (75.0)	02 (66.7)
Age (Years)										
≤ 10	14	00 (-)	08(15.1)	02 (09.1)	00 (-)	00 (-)	01 (07.1)	02 (25.0)	00 (-)	01 (33.3)
>10- 19	12	00 (-)	04 (07.5)	04 (18.2)	02 (22.2)	02 (07.4)	00 (-)	00 (-)	00 (-)	00 (-)
>20- 59	65	03 (100)	17 (32.1)	12 (54.5)	06 (66.7)	14 (51.9)	06 (42.9)	03 (37.5)	03 (75.0)	01 (33.3)
≥ 60	52	00 (-)	24 (45.3)	04 (18.2)	01 (11.1)	11 (40.7)	07 (50.0)	03 (37.5)	01 (25.0)	01 (33.3)
Clinical date										
Splenomegaly	118	03 (100)	45 (84.9)	19 (86.4)	00 (-)	26 (96.3)	14 (100)	07 (87.5)	02 (50.0)	02 (66.7)
Hepatomegaly	99	03 (100)	39 (73.6)	16 (72.7)	02 (22.2)	17 (63.0)	13 (92.9)	05 (62.5)	03 (75.0)	01 (33.3)
Bone pain	40	03 (100)	13 (24.5)	07 (31.8)	00 (-)	05 (18.5)	05 (35.7)	04 (50.0)	02 (50.0)	01 (33.3)
Fever	25	03 (100)	15 (28.3)	02 (09.1)	00 (-)	01 (03.7)	01 (07.1)	02 (25.0)	00 (-)	01 (33.3)
Bleeding	21	01 (33.3)	05 (09.4)	00 (-)	09 (100)	02 (07.4)	04 (28.6)	00 (-)	00 (-)	00 (-)
Gingival hypertrophy	19	00 (-)	00 (-)	00 (-)	00 (-)	04 (14.8)	10 (71.4)	05 (62.5)	00 (-)	00 (-)
Tumor mass	15	00 (-)	00 (-)	00 (-)	00 (-)	03 (11.1)	11 (78.6)	01 (12.5)	00 (-)	00 (-)
Chloroma	09	00 (-)	03 (05.7)	04 (18.2)	00 (-)	02 (07.4)	00 (-)	00 (-)	00 (-)	00 (-)
Lymphadenopathy	08	00 (-)	05 (09.4)	01 (04.5)	00 (-)	01 (03.7)	01 (07.1)	00 (-)	00 (-)	00 (-)

Note: (FAB) French American [3-5]

Table 3: Correlation between hematological date and FAB subgroups M0 to M7 of patients with acute myeloid leukemia.

Date	Patients n=143	AML-M0 n= 03 n+ (%)	AML-M1 n= 53 n+ (%)	AML-M2 n= 22 n+ (%)	AML-M3 n= 09 n+ (%)	AML-M4 n= 27 n+ (%)	AML-M5a n= 14 n+ (%)	AML-M5b n= 08 n+ (%)	AML-M6 n= 04 n+ (%)	AML-M7 n= 03 n+ (%)
B12 vitamin (pg/mL)										
≤ 200	Ur	Ur	Ur	Ur	Ur	Ur	Ur	Ur	00 (-)	Ur
> 200	Ur	Ur	Ur	Ur	Ur	Ur	Ur	Ur	04 (100)	Ur
+ Cytochemistry (SBB/MPO)	132	00 (-)	53 (100)	22 (100)	09 (100)	27 (100)	13 (92.9)	08 (100)	* 00 (-)	*00 (-)
WBC /PB(x 10³/μL)										
≤ 5.0	10	00 (-)	01 (01.9)	00 (-)	08 (88.9)	00 (-)	00 (-)	00 (-)	01 (25.0)	00 (-)
>5.0-10	11	00 (-)	01 (01.9)	05 (22.7)	00 (-)	02 (07.4)	01 (07.1)	00 (-)	01 (25.0)	01 (33.3)
> 10 - 50	40	00 (-)	16 (30.2)	06 (27.2)	00 (-)	11 (40.7)	03 (21.4)	01 (12.5)	00 (-)	02 (66.7)
> 50	82	03 (100)	36 (67.9)	11 (50.0)	01 (11.1)	14 (51.9)	10 (71.4)	06 (75.0)	02 (50.0)	01 (33.3)
% Blastic cells (PB)										
≤ 50	26	00 (-)	12 (22.6)	04 (18.2)	01 (11.1)	04 (14.8)	01 (07.1)	02 (25.0)	01 (25.0)	01(33.3)
>50	117	03 (100)	41 (77.4)	18 (81.8)	08 (88.9)	23 (85.2)	13 (92.9)	06 (75.0)	03 (75.0)	02 (66.7)
Platelet count/ PB (x 10³/μL)										
≤ 20	17	00 (-)	04 (07.5)	00 (-)	08 (88.9)	02 (07.4)	02 (14.3)	01 (12.5)	00 (-)	00 (-)
>20- 50	63	02 (66.7)	31 (58.5)	11 (50.0)	01 (11.1)	08 (29.6)	06 (42.8)	01 (12.5)	03 (75.0)	00 (-)
50 - 100	53	01 (33.3)	15 (28.3)	10 (45.5)	00 (-)	15 (55.6)	05 (37.1)	04 (50.0)	01 (25.0)	02 (66.7)
> 100	10	00 (-)	03 (05.7)	01 (04.5)	00 (-)	02 (07.4)	01 (07.1)	02 (25.0)	00 (-)	01 (33.3)
Hemoglobin (g/dL)										
< 10.0	107	02 (66.7)	45 (84.9)	13 (59.1)	08 (88.9)	16 (59.3)	11 (78.6)	06 (75.0)	03 (75.0)	03 (100)
10.0 - 12.0	33	01 (33.3)	08 (15.1)	08 (36.4)	01 (11.1)	09(33.3)	03 (21.4)	02 (25.0)	01 (25.4)	00 (-)
> 12.0	03	00 (-)	00 (-)	01 (04.5)	00 (-)	02 (07.4)	00 (-)	00 (-)	00 (-)	00 (-)

Note: (FAB) French American British classification 3-5; (+) Positive Reaction; (n) number of patients tested; (n+) number of positive cases; (%) percentage of positive cases; (PB), peripheral blood; (WBC) white cell count; (SBB) Sudan Black B; (MPO) Myeloperoxidase; (*) SBB/MPO staining negative in erythroid and megakaryocyte population; (Ur)unrealized.

Table 4: Diagnosis and classification of AML based on reactivity with various monoclonal antibodies.

AcMo	Patients n=143 n+ (%)	AML-M0 n= 03 n+ (%)	AML-M1 n= 53 n+ (%)	AML-M2 n= 22 n+ (%)	AML-M3 n= 09 n+ (%)	AML-M4 n= 27 n+ (%)	AML-M5a n= 14 n+ (%)	AML-M5b n= 08 n+ (%)	AML-M6 n= 04 n+ (%)	AML-M7 n= 03 n+ (%)
CD45	138 (96.5)	03 (100)	53 (100)	22 (100)	09 (100)	27 (100)	14 (100)	08 (100)	00 (-)	01 (33.3)
HLADR	120 (83.9)	03 (100)	53 (100)	22 (100)	00 (-)	27 (100)	14 (100)	08 (100)	02 (50.0)	01 (33.3)
CD34	106 (74.1)	03 (100)	53 (100)	12(54.5)	00 (-)	18 (66.7)	14 (100)	01 (12.5)	04 (100)	01 (33.3)
CD117	131 (91.6)	02 (66.7)	53 (100)	20 (90.1)	09 (100)	27 (100)	13 (92.9)	05 (62.5)	02 (50.0)	01 (33.3)
anti-MPO	124 (86.7)	03 (100)	53 (100)	10 (100)	09 (100)	27 (100)	05 (35.7)	08 (100)	00 (-)	00 (-)
CD13	130 (90.1)	03 (100)*	53 (100)	22 (100)	09 (100)	27 (100)	14 (100)	07 (87.5)	00 (-)	00 (-)
CD33	139 (97.2)	01 (33.3)	53 (100)	22 (100)	09 (100)	26 (96.3)	14 (100)	08 (100)	04 (100)	03 (100)
CD36	44 (30.8)	00 (-)	00 (-)	00 (-)	00 (-)	25 (92.6)	09 (64.3)	06 (37.5)	04 (100)	03 (100)
CD14	38 (26.6)	00 (-)	00 (-)	00 (-)	00 (-)	22 (81.5)	10 (71.4)	06 (75.0)	00 (-)	00 (-)
CD64	46 (32.2)	00 (-)	00 (-)	00 (-)	00 (-)	26 (96.3)	14 (100)	06 (75.0)	00 (-)	00 (-)
CD65	118 (82.5)	00 (-)	53 (100)	22 (100)	09 (100)	27 (100)	07 (50.0)	00 (-)	00 (-)	00 (-)
CD66b	49 (34.3)	00 (-)	00 (-)	22 (100)	00 (-)	27 (100)	00 (-)	00 (-)	00 (-)	00 (-)
CD71	42 (29.4)	03 (100)	20 (37.7)	05 (22.7)	00 (-)	00 (-)	05 (35.7)	00 (-)	04 (100)	00 (-)
CD235a	04 (02.8)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	04 (100)	00 (-)
CD41	03 (02.1)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	03 (100)
CD42b	03 (02.1)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	03 (100)
CD61	03 (02.1)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	03 (100)
CD1a	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)
CD2	10 (06.7)	00 (-)	04 (02.0)	04 (02.0)	00 (-)	02 (07.4)	00 (-)	00 (-)	00 (-)	00 (-)
CD3	08 (05.6)	00 (-)	02 (03.8)	03 (13.6)	00 (-)	02 (07.4)	01 (07.1)	00 (-)	00 (-)	00 (-)
CD3+/CD4+	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)
CD3+/CD8+	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)
CD4	35 (24.5)	00 (-)	00 (-)	00 (-)	00 (-)	20 (70.1)	11 (78.6)	04 (50.0)	00 (-)	00 (-)
CD7	32 (22.4)	02 (66.7)	20 (37.7)	05 (22.7)	00 (-)	03 (11.1)	01 (07.1)	00 (-)	00 (-)	01 (33.3)
CD56	23 (16.1)	00 (-)	03 (05.7)	11 (50.0)	02 (22.2)	00 (-)	05 (35.7)	02 (25.0)	00 (-)	00 (-)
CD10	01 (0.70)	00 (-)	01 (01.9)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)
CD19	12 (08.4)	00 (-)	02 (03.8)	09 (40.9)	00 (-)	01 (03.7)	00 (-)	00 (-)	00 (-)	00 (-)
CD20	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)
CD22	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)
cytCD79a	04 (02.8)	00 (-)	02 (03.8)	00 (-)	00 (-)	00 (-)	02 (-)	02 (25.0)	00 (-)	00 (-)
IgM	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)
nuTdT	04 (02.8)	00 (-)	04 (07.5)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)	00 (-)

Note: (FAB) French American British classification 3-5; (n) number of patients tested; (n+) number of positive cases; (%) percentage of positive cases; (HLADR) Type II major histocompatibility complex; (anti-MPO) AcMo against myeloperoxidase; (CD13* or cytCD13) Intracytoplasmic CD13; (CD3+/CD4+) T-helper lymphocytes; (CD3+/CD8+) T-cytotoxic lymphocytes; (cytCD79a) Intracytoplasmic CD79a; (nuTdT) nuclear Terminal deoxynucleotidil Transferase.

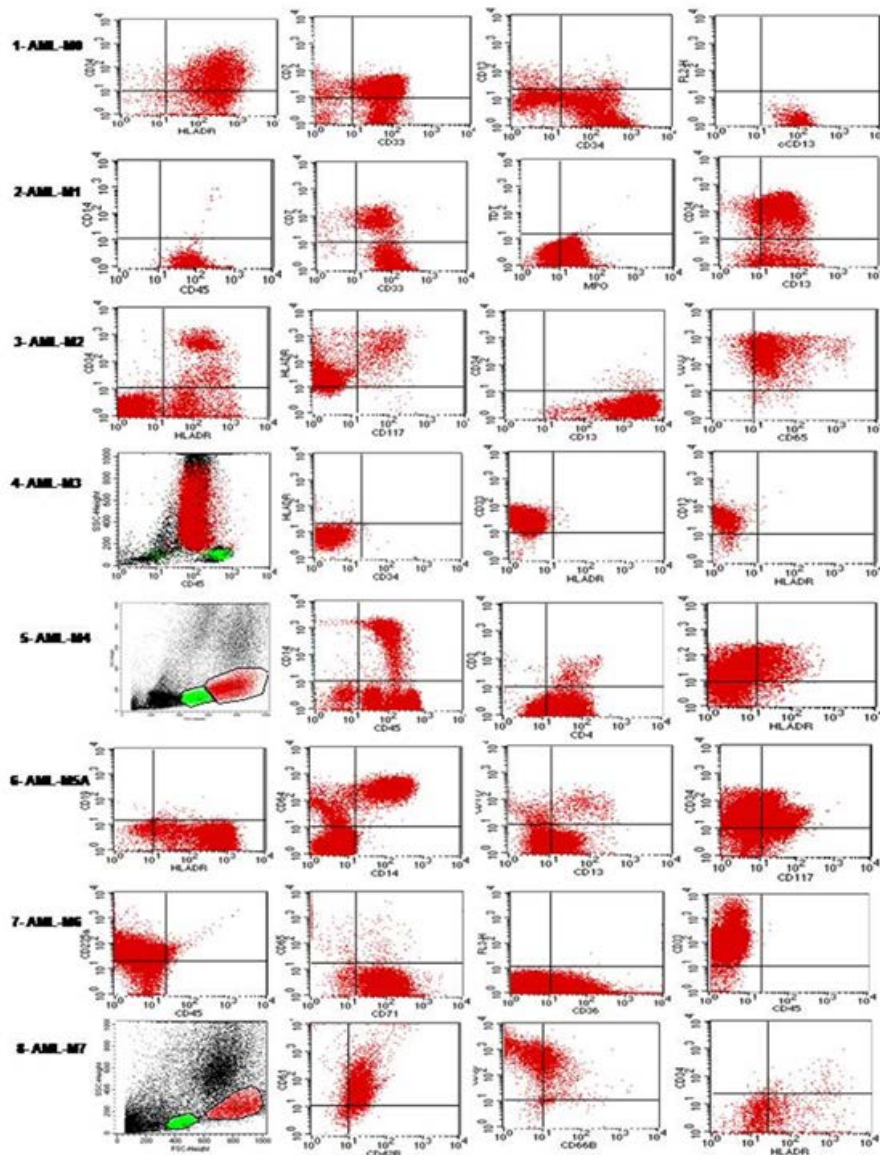


Figure 1 Identification leukemia-associated immunophenotype patterns (LAIP) of in subgroup of AML.
Note: 1-AML-M0; 2-AML-M1; 3-AML-M2; 4-AML-M3; 5-AML-M4; 6-AML-M5A; 7-AML-M6; 8-AML-M7.

and HLADR (**Figure 1**). Monocytic AML (M4/M5), expression of CD14 and CD64, associated with CD13, CD33 and HLADR (**Figures 1**).

AML-M6 was identified by strong expression of CD235a and CD71, associated with CD33, CD36 and CD34 (**Figure 1**). In these AML, serum levels of vitamin B12 were normal, ruling out the diagnosis of MA.

AML-M7 was confirmed by the expression of platelet glycoproteins CD41, CD42b and CD61 (**Figure 1**).

Aberrant expression of lymphoid antigens has been observed in some cases, especially CD4, CD7 and CD56. The CD4 was observed in 24.5% of cases, more present in cases of AML-M4/M5. CD7 was positive in 22.4% of cases, with more expressive values in AML-M0/M1 and CD56 was expressed in 16.1% of cases, notably in the subtypes AML-M2, M5a/b and M3. Other

lymphoid antigens such as TdT, CD3, CD10, CD19, CD2 and CD79a were observed in less frequently.

Discussion

For the diagnosis and classification of AML, the World Health Organization (WHO) recommends careful integration of clinical history, morphology analysis of BM and PB smears, cytogenetic/molecular genetic analysis and immunophenotyping [24]. However, cytogenetic/molecular analyzes may not be available in many services, and cytomorphological analysis complemented by immunophenotyping is satisfactory for rapid diagnosis and classification of this leukemias [25].

It is important to identify the subtype of AML, as this influences the choice of treatment type and the patient's prognosis [26]. For example, in the case of APL, in which the diagnosis is clinically

urgent, the combination of these two modalities permits a diagnosis with high precision [1,16,26]. In some other types of AML, immunophenotypic features may provide an indication as to the cytogenetic anomaly likely to be present [1, 6,17,18,26].

Highlighting the AML with t(8;21) observed in immunophenotype cases with strong CD34+, HLADR+, weak CD33+, MPO+, CD65+ or CD15+ and IL-2 receptor (CD25+) can observe aberrant expression for lymphoid antigens CD19, CD79a, TdT and CD56. In AML, chromosome 16 inversion correlates with expression of CD34, CD117 and myeloid subpopulations with MPO+, CD13+, CD33+, CD15+, CD65+; AML with a monocytic component with expression of CD11b, CD11c, CD64, CD36 and CD14 and aberrant expression of CD4 AND CD2. AML with t(9;11) 11q23 correlates with aberrant expression of the lymphoid antigens CD19 and CD2 [1,6,-18,26].

Detection of MPO is probably the most specific technique for differentiating between myeloid and lymphoid lineages, which can be done by cytochemical methods or immunophenotyping [16,25]. It is a lysosomal enzyme found in the primary granules of cells in the myeloid series [26]. Its expression occurs at the beginning of myeloid differentiation and appears to be specific for cells of that lineage. MPO has never been reported in ALL [2,7,27,28].

Most studies have found a higher incidence of AML in males, although this predominance is not as distinct as in ALL [1]. In our series, a male prevalence was present, with a male to female ratio of 1.4 to 1 (**Table 2**).

Most patients had pallor and fatigue, possibly due to anemia [1]. In present study, splenomegaly and hepatomegaly were the most common clinical findings. Bleeding was more present in patients with APL (**Table 2**).

In the immunophenotypic evaluation of AL, the expression of one or more pan-myeloid antigens such as CD13, CD33, CD65, CD117 and MPO are sufficient for the diagnosis of AML [1,13,17-19,26].

Furthermore, the immunophenotypic classification has diagnostic and prognostic importance in some subtypes of AML. Thus, it is essential for the diagnosis of AML-M0 and M7, being helpful in the diagnosis of the variant hypogranular form of APL (AML-M3v), in the differentiation between subgroups AML-M1 and M2, in subtypes AML-M5a and M5b, and in the differential diagnosis between AML-M6 and MA [6,13-15,18,19,25].

The AML-M0 is characterized by the infiltration of BM by blast cells with negative cytochemical reaction for MPO and SBB. Blasts cells are small, with loose chromatin and evident nucleoli, presenting agranular cytoplasm, without Auer bodies [2-4].

Immunophenotyping shows a blast population with a low FSC/SSC ratio, with positivity for at least one of the myeloid antigens such as CD33, cytCD13, CD117 and CD34 [24-25]. Lymphoid lineage antigens are generally negative, although CD7 and CD56 are observed in some cases [1,25]. As the therapeutic approach to AML differs from ALL, it is important to perform immunophenotyping in the differentiation between AML-M0 from ALL and the consequent correct treatment guidance [1,25].

The AML-M1 is associated with the expression of CD13, CD33, CD34, CD65, CD117 and HLADR in variable combinations [25]. In this AML group, the co-expression of CD34 and HLADR is significantly greater than that observed in the AML groups with maturation as M2/M3 [1,6,13,17,18,25].

AML-M2 is characterized by the presence of >30% of myeloblasts associated with 10% of mature granulocytes in BM. Blast cells of a large size, with abundant and basophilic cytoplasm, often containing azurophilic granules. Auer bodies are frequent [3-4]. Immunophenotyping leukemic cells exhibit CD65, CD66, HLADR, CD13 and CD33, but CD34 expression is very weak or sometimes may be absent [1,6,13,18,19,25]. Aberrant expression of lymphoid antigens CD19, CD2, CD7 and natural killer cells (CD56) can also be observed, as these are associated with t(8:21) and are related to good prognosis and higher rates of complete prolonged remission in adult patients [25].

APL is defined by the proliferation of leukemic promyelocytes in BM, which have a large nucleus, and a cytoplasm with many coarse granulations. In some cases, the presence of numerous Auer bodies "faggot cell" is observed [3-4,16]. In AML-M3v, promyelocytes have a large and convoluted nucleus. The cytoplasm is basophilic with little or no granulation [3,4]. Cytochemical stains for MPO and SBB are strongly positive in both types of APL [16].

The immunophenotypic reveals high auto fluorescence and higher FSC/SSC ratio [24] and positivity to MPO, CD13, CD33 and CD117, but there is a lack of CD34 and HLADR [15,17,18,25]. These immunophenotypic, clinical and hematological characteristics of APL were also found in the present study (Tables 2-4 and Figure 1).

In acute myelomonocytic leukemia (AML-M4), leukemic cells exhibit monocytic and granulocyte precursors [3-4]. Monocytic precursors constitute >20% of the nucleated cells in the BM, representing about 12% of AML [3-4]. The variant eosinophilic form (AML-M4eo) can be found, with an increase in the number of eosinophils related with chromosome 16 abnormalities, either inv(16)(p13q22) or chromosome 16 inversion, being associated with a better prognosis and response to treatment [14,17,18,25].

In FC immunophenotyping, two distinct populations of leukemic cells are typically observed based on the FSC/SSC pattern, one of a large size (high FSC) and a smaller one (low FSC), corresponding to blastic cells from monocyte and granulocyte lineage, respectively [24]. Monocyte antigens CD14 and CD64 are positive along with HLADR, CD11b, CD11c and CD36. Myeloid antigens CD13 and CD33 are generally positive [14,17,18,25]. The AML-M4eo, exhibits aberrant expression of the CD2 lymphoid line antigen may occur and weak expression of the CD4 antigen can be observed in the monocyte population [14,7-18,25].

Monocytic Leukemias (AML-M5) are defined when 80% or more of non-erythroid cells in the BM are composed of monoblasts, promonocytes or monocytes. The AML-M5a subtype has >80% of monoblasts, while the LMA-M5b subtype has a predominance of promonocytes [2-4].

The immunophenotypic profile characteristic of AML-M5 is the presence of a leukemic population with a higher FSC/SSC ratio than in AML subgroups M0, M1 and M2 [24]. Monocytes antigens

CD14 and CD64 are positive along with HLADR, CD11b, CD11c [14,25]. Elevated levels of CD14 expression is a specific feature of mature monocytes, being often absent or underexpressed in immature monocytic cells [14]. In addition, other antigens that are generally considered to be associated with monocytes may be expressed in other types of AML, such as CD33 and CD13 [14,25]. The CD34 is generally negative, being more present in the most immature subgroup (AML-M5a) [1,14,17,18,25].

The CD36 is not specific and can also be seen in the erythroid, megakaryocytic and monocytic series [25]. The weak expression of the CD4 antigen can be observed [14,25]. AML-M5 with CD33 and CD4 expression associated with negativity to CD13 and CD34 is frequently correlated with t(9;11) [1,14,25].

Patients with monocytic leukemia have a high incidence of extra-medullary disease, with infiltration in the gums, skin, digestive tract and CNS. The presence of hepato-splenomegaly and leukocytosis is more frequent compared to other FAB subtypes [14]. These characteristics are consistent with the clinical, hematological and immunophenotyping data of AML-M5a/M5b observed in the present study (Tables 2-4).

Erythroleukemia (AML-M6) is a rare type of AL, corresponding to 2 to 3% of AML, defined by $\geq 50\%$ of the nucleated cells in the BM being of erythroid origin [3-4]. Two subtypes herein described AML-M6a (erythroid/myeloid AML) with the presence of leukemic components of erythroid and myeloid origin, and AML-M6b (pure erythroid AML), characterized by the presence of 80% or more of erythroid precursors in BM, and are rarely seen and clinically more severe than AML-M6a [26]. Cytochemical reactions of MPO and SBB are negative in leukemic erythroid cells [16].

AML-M6 can be differentiated from AM by immunophenotyping, being made by the expression of CD235a, CD36 and strong expression of CD71 in erythroid lineage cells [25]. Investigation of CD33, CD117 and CD34 may also be useful, as they are negative in MA and generally positive in erythroid cells of AML-M6. The phenotype of the myeloid population is similar to that observed in AML-M0/M1 [13,18,19,28,29]. Additionally, vitamin B12 dosage may be useful in diagnostic complementation, with low or absent serum levels in MA. These characteristics of these AML were also observed in the present study (Tables 2-4).

Transferrin receptor (CD71) is an integral membrane protein encoded by a gene localized in chromosome 3 that mediates cellular iron uptake by the erythroid lineage for hemoglobin synthesis and may actively proliferate cell population, since iron is required for cell division. It is expressed by a wide variety of cells as erythrocyte precursors [30].

In AML, CD71 expression correlates with proliferative activity of leukemic cells, with consensus on the co-expression of CD71 and CD34 used to stratify AML patients as a poor prognostic factor [30]. In present investigation, CD71 expression was found in 29.4% of all cases, being significantly higher in more immature AML and all cases of AML-M6.

Acute Megakaryoblastic Leukemia (AML-M7) is defined by the presence of more than 30% of megakaryoblasts among nucleated cells [3-4]. This leukemia is rare, but relatively frequent in children with Down syndrome [28-29].

AML-M7 can be confused with ALL or AML-M0 by morphological criteria. Blast cells from BM are small, pleomorphic, basophilic cytoplasm without granules, with projections (blebs) and negative for cytochemical stains such as MPO and SBB [3-4].

The main immunophenotypic characteristic of blastic cells from AML-M7 is the expression of platelet antigens CD41 (glycoprotein complex IIb/IIIa), CD42 (glycoprotein Ib) and CD61 (glycoprotein IIIa). The expression of pan-myeloid antigens CD13 and CD33 is described, as has positive cases of CD36. Some cases can be HLADR and CD34 negative [13,8-19,26]. These immunophenotyping of AML-M7 characteristics were also observed in the present study (Table 4).

In addition to the distinction between AML and ALL and AL classification, immunophenotyping allowed the identification of additional prognostic factors, allowing a better stratification into risk groups, enabling a differentiated therapeutic approach. Positivity to CD34, CD71, aberrant expression of lymphoid antigens and Multidrug Resistance (MDR) phenotype are highlighted as factors of poor prognosis of AML [1,8,19,25,27,30-32].

CD34 is a 105-120 KD glycoprotein expressed on many different cell types, more specifically in immature hematopoietic stem cells mediating the binding of hematopoietic stem cells to extracellular matrix proteins or stromal cells in the precursors B and T lymphocytes and even more immature Colony-Forming Cells (CFUs) and myeloblastic [31].

In AML, CD34 has in 45-68% of cases with a higher incidence in more immature subtypes us M0, M1 and M5a, and this expression of has adverse prognostic factors, such as a higher recurrence rate and refractory disease [6,31].

Some authors have reported that the prognostic significance of CD34 expression increases if co-expression occurs with other antigens such as HLADR [6], CD71 [30] and aberrant expression of CD7 [31-36]. A high intensity of co-expression of CD34 together with CD19 and CD56 is characteristic of AML-M2 with t(8;21) translocation [17,18].

It has also been reported that among AML patients co-expressing CD34 together with MDR proteins such as P-glycoprotein (Pgp) and multidrug resistance-associated protein-1 (MRP1) they was a statistically significant lower rate of complete remission or shorter overall survival [19].

In present study, we found CD34 positivity in 106 cases of AML, with more expressive levels in AML with very immature blasts (M0, M1 and M5a), in contrast to groups M2, M5b, which showed lower levels of positivity and negativity in all cases of AML-M3 (Table 4).

Aberrant phenotypes are associated with AL, identified by the co-expression of cell markers that are rarely or never found simultaneously in normal hematopoietic differentiation, overexpression of a specific cell line marker or absence of a marker, which configures the maturation asynchronism of a cell line [1,18,19,25,36-38].

Some immunological and molecular studies have reported that many AL may present antigenic characteristics of more than one

cell line, characterizing two groups of leukemias that presented "lineage infidelity", that is, ALL expressing antigens associated with the myeloid lineage and AML expressing antigens of lymphoid lineage [1,8,13,36-38].

However, it is important to make a distinction between biphenotypic leukemia with ALL or AML with aberrant expression of markers from other strains, due to differences in therapeutic approaches, therefore it is recommended to investigate the scoring system suggested by the EGIL group [13], as well as the use of a panel made up of multiple MoAb combined with different fluorochromes, which makes it possible to investigate the expression of different antigens in the same cell by means of FC [17,18].

The mechanisms by which the expression of aberrant phenotypes occurs in the development of LAs remain unclear, however, it is possible to establish associations between these unusual expressions and other biological characteristics of the disease, such as associations with chromosomal translocations and adverse prognostic factors [17,18,25,30,32-36].

According to the WHO, several lymphoid immunophenotypic cell markers may be aberrantly expressed in AML [25,26]. TdT may be expressed in greater than one-third of cases, CD7, CD2, CD19 and CD56 may be expressed frequently; however, the T-cell antigen CD3 is usually absent [1,18,19,25].

TdT is a nuclear polymerase normally expressed during the early stages of B and T cell differentiation [37]. In initial reports, TdT expression was believed to be limited to ALL and can account in 18-24% of AML, more frequently in subtype M0 and M1. No specific chromosomal abnormalities were associated with AML TdT+ 37.

CD7 is a 40kDa glycoprotein encoded by a gene situated on chromosome 17 [33]. In T-cells CD7 play an important role in the cellular activation. Some authors, however, believe that the expression of this antigen in T progenitor cells could be related to mediating the migration of these cells from the MB to the thymus. It is identified in hematopoietic progenitor cells that can give rise to other cell lines and can thus be observed in AML more frequently in subtypes M0 and M1 [33].

According to some authors, the co-expression of CD7 with other cellular markers related to poor prognosis in AMLs such as CD34, Pgp and MRP1 [19]. In our investigation, CD7 expression was observed in 32/143 patients, most of whom had CD34 co-expression, characterized AML very immature (Table 4).

CD2 is a 45-58kD glycoprotein present on the surface of T lymphocytes and natural-killer cells (NK) and is not normally expressed in human myeloid cells, but is found in a significant minority of AML cases, M2, M3v, M4 and M5 groups [17,18,25,36]. Expression of CD2 and other T-lymphoid antigens such as CD4, CD7 and CD56 in myeloid blast cells are correlated with extramedullary disease [17,18,25,36].

CD19 is a 95kD glycoprotein, which appears very early during the maturation of B-lymphocyte precursors and is constitutively expressed in mature normal B-lymphocytes and related neoplasm but not in plasma cells [32,35]. CD19 expression has

been observed in 2-22% of AML cases commonly associated with AML-M2 with translocation t(8;21) and t(8;19) and also in AML subgroups M3v, M4 and M5 [17,18,25]. In the present study, CD19 and CD2 expression were observed in 8.4% and 6.7% of cases respectively, with a predominance of CD19 in M2 and CD2 in M3 subgroup of AML (Table 3).

Neural cell adhesion molecule (NCAM or CD56) is a 180 kD glycoprotein, encoded by a gene located in chromosome 11, and it is expressed on most normal NK cells [38]. In addition, CD56 expression was found in rare subsets of T-lymphocytes (NKT cell), dendritic cells, and neural and mesenchymal stem cells [8]. Aberrant expression of CD56 in AML is present in 13-29% of cases with high frequency in AML M2, M3 and M5. It identifies a subset of patients with a bad prognosis as extramedullary involvement and high leukocyte count in the AML-M2 with t(8;21) and t(15;17) [38-40]. In our samples, CD56 expression was observed in 23 cases with predominance in AML-M2 cases (Table 4).

Conclusion

We conclude that the immunophenotypic patterns observed in patients with AML allowed the accurate identification of different groups of this leukemia. Additionally, through CF we also identify varied patterns of aberrant phenotypes that also contribute to the diagnosis and identification of prognostic factors.

Author Contributions

Linduarte V. Morais, Taissa Maria M. Oliveira, Erica A. Gil, Lenilton S Silveira Jr, Victor L Soares and Rafael L Duarte, collected the data and contributed to the writing of the manuscript. Dany Kramer, Aldair S Paiva and Gustavo Oliveira contributed to the clinical interpretation of laboratory analysis. Geraldo B. Cavalcanti Jr conceived and conducted the study, contributed clinical and flow cytometry data, and reviewed the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest of any kind.

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