

Multiparametric flow cytometry immunophenotyping in acute leukemia: A nationwide single centre study in Albania

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Abstract

Aim: Our aim was to determine the diagnostic relevance of flow cytometry multiparametric immunophenotyping (FCMI) in acute leukemias (AL) of all ages in Albania.

Methods: A total of 300 bone marrow or peripheral blood samples of patients suspected for AL were examined in Tirana, Albania. The applied methodology consisted of a four color FCMI method.

Results: Of the overall 300 patients studied, 128 (42.52%) were females and 172 (57.47%) were males. There were 103 (34.55%) children aged 0-16 years, whereas further 197 (65.44%) were adult patients. After the FCMI examination, 78 (75.7%) of the pediatric patients were diagnosed with acute lymphoid leukemia (ALL). In this group, 78.2% of the patients showed a malignant proliferation of B-cell line (ALL-B) and 21.8% of T-cell line (ALL-T). About 21% of the pediatric patients resulted with acute myeloid leukemia (AML). One patient displayed acute bilineal leukemia (BLL) and another patient biphenotypic acute leukemia (BAL). Only one patient displayed undifferentiated immunophenotypic cells. From the 197 adult patients with AL studied, we established the following immunophenotypic diagnosis: 139 (70.5%) of them were diagnosed with AML and 53 (26.9%) with ALL. In the latter group, 77.5% of the patients showed an ALL-B and 22.6% presented an ALL-T cell proliferation. Three adult patients were classified as BAL, while two of them displayed an undifferentiated immunophenotypic profile because of the expression of only non-specific cell line markers CD45, CD34 and CD117.

Conclusion: Flow cytometry immunophenotyping is a very important examination for the final diagnosis and subtype differentiation of AL also in Albanian patients.

Keywords: acute leukemia, flow cytometry, immunophenotyping, monoclonal antibodies.

Introduction

Acute leukemias (AL) represent biologically and clinically heterogeneous conditions of malignant clonal proliferations of haematopoietic progenitor cells (1). They are characterized by the proliferation and accumulation of abnormal leukemic cells, blocked at different levels of differentiation and along different cell lineages (2). The specific causes are not clear, but some risk factors and chromosomal anomalies have been identified. Among the risk factors are mentioned the exposure to ionizing radiation and some chemical substances, previous treatments with chemotherapy, exposure to various viral infections, as well as the genetic predisposition (1,3). The malignant transformation occurs in the bone marrow and can affect any cell line. Acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) are the more frequent subtypes, but any hematopoietic cell line can be affected from the malignant transformation (1,4). The actual development of effective therapies has made possible the reduction of the disease-related mortality, the increase of the complete long-term remission and the improvement of the disease-free survival rates for AL (5). In this context, the accurate diagnosis of various AML and ALL subtypes is of essential value for their classification, hence playing a crucial role for individual risk stratification and therapy establishment (6).

AL diagnosis is based on a series of biological examinations. The cytological methods, carried out through peripheral blood and bone marrow cell morphology examination remains still the first diagnostic step, but in many cases it is difficult to distinguish morphologically the premature blast cells, since there is a great similarity between early precursors of different cell lines (7). In these cases, further cytochemical examinations are required in order to determine the proper cell line, particularly when there is an asynchronization between nuclear and cytoplasmic maturation. However, the morphological and cytochemical characteristics of the abnormal cells do not always allow a preliminary determination of the cell type line involved in the

malignant proliferation. Therefore, the cytomorphic examination should always be complemented by immunophenotyping techniques and by further cytogenetic and molecular biology methods (8). Flow cytometry multiparametric immunophenotyping (FCMI) is a very important diagnostic step for the accurate biological diagnosis of AL (9). This method is based principally on the different expression of antigens on the cell surface or cytoplasm. These molecules serve as specific markers for a particular cell line and for the differentiation stage of this line. A precise and simultaneous study of these markers enables the fine diagnosing of different types of malignant hemopathies (10). FCMI plays an important role also for the prognostic evaluation and minimal residual disease monitoring (11). Finally, this method remains the best option for a rapid and accurate diagnosis. In this context, we studied the role of FCMI in AL subtype differentiation, in terms of a single center for this purpose in Albania, a former communist country in the Western Balkans.

Methods

Patients and samples studied

This study was carried out at the Laboratory of Immunology and Tissue Typing of the University Hospital Center "Mother Teresa" in Tirana, during the time interval 2009-2013. During this period, 300 consecutive samples from suspected and not yet treated AL patients were studied through FCMI. Of these, 180 were bone marrow samples, 119 peripheral blood samples and in one patient a cerebrospinal fluid (CSF) sample was collected. Anticoagulant K3EDTA tubes were used for the biological sample collection.

Flow cytometry multiparametric immunophenotyping methodology

The applied methodology was a four color FCMI technique that was performed in an EPICS-XL Beckman-Coulter flow cytometer (Coulter Epics XL-MCL, CA, USA). This instrument measures simultaneously Forward Scatter (FS - distribution of light in a straight line), and Side Scatter (SS-

distribution of light at the right angle), which provide data for cell size and cell granularity respectively. At the same time the instrument enables the measurement of four different fluorescent dyes (FITC, PE, ECD and PC5) (12).

The monoclonal fluorescent antibodies used were as following: anti-CD3-PC5, CD4-PE, CD5-PE, CD7-PC5, CD8-ECD, CD10-PC5, CD13-PE, CD14-PC5, CD15-PC5, CD16-PE, CD19-ECD, PC5, PE, CD33-PC5, CD34-PE, ECD, CD45-FITC, CD56-PE, CD64-PE, CD79a-PC5, CD117-PC5, MPO-PE and HLA-DR-ECD.

Four-color panels were used with different cocktail fluorochrome-conjugated antibodies. In each panel the CD45-FITC marker was used for "gating" the normal and abnormal cell populations studied. For the examination of the intra-cytoplasmic antigens, the panel used is CD45-FITC/cMPO-PE /cCD3-ECD/ cCD79a-PC5. The threshold border of the monoclonal antibody positivity for the detected abnormal blast cells was established at over 20% of the total cells studied (9,11).

Immunophenotypic criteria for AL subtype diagnosis

For AL subtype classification, we used the system proposed by the European Group for the Immunological Characterization of Leukemia (EGIL) (4).

This system is based on the number and the intensity of specific antigen expression on the leukemic lymphoid/myeloid cell lines.

Statistical analysis

The GraphPad software (GraphPad Software, Inc. La Jolla, CA USA) was used in order to evaluate the differences between categorical variables through the Fischer's exact test and also for the elaboration of continuous data.

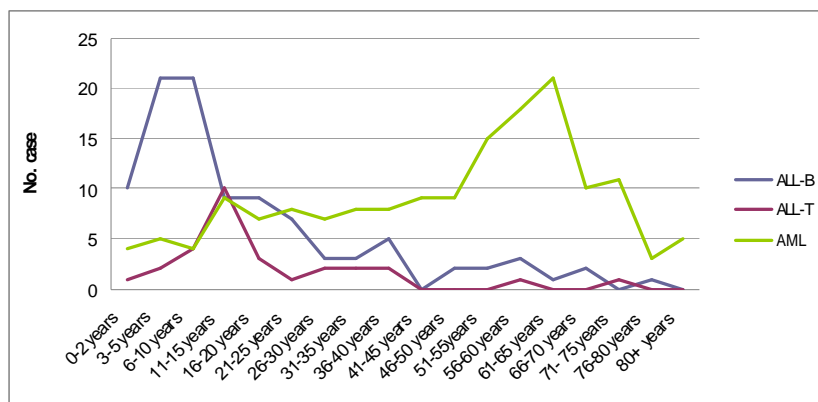
Results

General data about the patients studied

From all the 300 patients studied, 128 (42.52%) of them were females and 172 (57.47%) were males, with a ratio of 1:1.34. There were 103 (34.55%) children aged 0 to 16 years (mean: 7.3 years, median: 6 years, 95%CI=6.42-8.17), while 197 (65.44%) were adults aged 17 to 81 years (mean: 47.0 years, median 50 years, 95%CI=44.43-49.65). In the group of 103 children, 40 (38.46%) of them were females and 63 (61.53%) were males. In the group of 197 adults, 109 (55.32%) of them were males and 88 (44.67%) were females. AL distribution by age and type is presented in Figure 1.

In general, ALL constituted 43.66% (131 patients) and AML 53.66% (161 patients) of all the 300 patients studied.

Figure 1. Presentation of acute leukemia types according to the age-groups and the cell line affected



Immunophenotypic diagnosis of AL in children

Considering the results obtained after performing the FCMI procedure (Table 1), 78 (75.7%) of the

103 patients aged 0 to 16 years resulted with acute lymphoid leukemia (ALL). In this group, 61 (78.2%) patients (mean: 6.26 years, median: 5

years, 95%CI=5.28-7.24), showed a B-lymphoid cell line (ALL-B) and 17 (21.8%) of them (mean: 10.4 years, median: 11 years, 95%CI=8.25-12.57) a T-lymphoid cell line proliferation (ALL-T). Based on the expression of cMPO, CD13 and CD33 markers and the lack of CD19, cCD79a and cCD3, 22 (21.35%) of all childhood patients resulted with acute myeloid leukemia (AML). Of these, a 4-year old male resulted with undifferentiated immunophenotypic cells (UAL - Undifferentiated Acute Leukemia) since it expressed only CD34 and CD117 markers, while another 15-year old child of male sex resulted with Bilineal Acute Leukemia (BLL). On the basis of the CD45/SS cell distribution, it presented two abnormal cell populations separated from each other, where one cell line expressed CD33, MPO and CD117 myeloid cell markers and the other population the cCD79a and CD10 lymphoid cell markers. CD34 marker was expressed in both

blast cell populations. Another 6-year old female child resulted with a biphenotypic cell line proliferation (BAL), since the abnormal cell population expressed the myeloid cell line MPO, CD13, CD34 and also the lymphoid cell line markers CD79a and CD10 simultaneously (Table 1).

The expression of cell markers in B-ALL in children

Concerning the specific B-cell markers, CD19 was present in 98.4% of the blast cell populations studied, while the intracellular CD79a cell marker resulted positive in 96.6% of the patients. CD10 marker was positive in 86.9% of the patients studied. Regarding the non-specific cell marker HLA-DR, it was positive in 91.5%, while CD34 was positive in 62.3% of the patients. CD45 resulted positive, but in low expression, in 64.5% of the patients.

Table 1. General characteristics of the pediatric age patients studied, classified according to their AL subtype differentiation after FCMI

	B-ALL*	T-ALL †	AML ‡	BAL §	BLL II	UAL ¶
Female sex No (%)	25 (40.9%)	4 (23.52%)	11(50%)	1	0	0
Male sex No (%)	36 (59.01%)	13 (76.47)	11 (50%)	0	1	1
Mean age (years)	6.26	10,4	8	4	15	6
Median age (years)	5	11	7.5			
95% CI (years)	5.3-7.2	8.2 -12.6	5.7-10.3			
Total No (%)	61 (59.2%)	17 (16.5%)	22 (21.3%)	1 (0.97%)	1 (0.97%)	1(0.97%)

* B-ALL Acute lymphoblastic leukemia B cell

†T-ALL Acute lymphoblastic leukemia T cell

‡AML Acute myeloblastic leukemia

§ BAL Biphenotypic acute leukemia

II BLL Bilineal acute leukemia

¶ UAL Undifferentiated acute leukemia

An aberrant expression of the myeloid-cell surface markers CD13 and CD33 was observed in the blast cell population of 4 (6.5%) of the patients studied (Table 2). The dominant immunophenotypic profile in B-ALL of pediatric age was CD19+CD10+CD34+cCD79a + in 59% of patients, followed by CD19+CD10+CD34-CD79a+ (29.5%) and CD19+CD10- CD34+ cCD79a+ (3.2%).

Cell markers in children T-ALL

In 17 T cell-ALL pediatric patients (13 [76,47%] males and 4 [23,52%] females), the mean age was 10.4 years, median 11 years, 95%CI=8.25-12.97 (Table 1). The specific marker with higher frequency was CD7 (94.1%). The classical T-lymphoid cell line markers positivity were as follows: cCD3 - 82.35%, CD5 - 76.47 %, CD3 - 35.29 %, CD4 - 47.1 % and CD8 - 52.94% (Table

Table 2. Summary matrix: marker frequency rates on abnormal cell populations of all AL patients according to their subtype differentiation after MCFI

CD marker	AML*			B-ALL†			T-ALL‡			BAL§		
	Adult N =139	Children N =22	SS	Adult N =41	Children N =61	SS	Adult N =12	Children N =17	SS	Adult N =3	Children N =2	SS
	% of positivity			% of positivity			% of positivity			% of positivity		
CD45	100.0	100.0	nss	82.9	64.5	p=0.045	100.0	100.0	nss	100.0	100.0	nss
CD34	56.83	50	nss	51.2	62.3	nss	33.3	11.8	nss	66.6	100.0	nss
CD117	57.5	40.1	nss	2.4	1.6	nss	0.0	0.0	nss	33.3	0.0	nss
HLA-DR	80.5	71.4	nss	79.8	91.5	nss	0.0	0.0	nss	66.6	100.0	nss
cMPO	81.3	81.3	nss	0.0	0.0	nss	0.0	0.0	nss	100.0	100.0	nss
CD13	86.3	77.3	nss	3.2	4.9	nss	0.0	5.8	nss	33.3	0.0	nss
CD33	82.7	72.3	nss	3.2	4.9	nss	8.4	5.8	nss	33.3	50.0	nss
CD14	11.2	9.1	nss	-	-	nss	-	-	nss	-	-	nss
CD15	2.3	4.5	nss	-	-	nss	-	-	nss	-	-	nss
CD16	2.1	4.5	nss	-	-	nss	-	-	nss	-	-	nss
CD64	22.4	13.6	nss	-	-	nss	-	-	nss	-	-	nss
CD19	0.7	9.1	p=0.008	92.8	98.3	nss	0.0	0.0	nss	66.6	50.0	nss
CD79a	0.0	0.0	nss	92.8	96.7	nss	0.0	0.0	nss	66.6	100.0	nss
CD10	0.0	0.0	nss	70.7	86.8	p=0.036	16.6	17.6	nss	33.3	100.0	nss
cCD3	7.46	0.0	nss	0.0	0.0	nss	83.3	82.3	nss	33.3	0.0	nss
CD3	4.3	9.1	nss	0.0	0.0	nss	50.0	35.3	nss	33.3	0.0	nss
CD4	9.4	13.6	nss	0.0	0.0	nss	58.3	47.1	nss	33.3	0.0	nss
CD5	-	-	nss	0.0	0.0	nss	83.3	76.5	nss	0.0	0.0	nss
CD7	5.2	4.5	nss	0.0	0.0	nss	91.6	94.1	nss	33.3	0.0	nss
CD8	0.0	0.0	nss	0.0	0.0	nss	41.6	52.9	nss	0.0	0.0	nss
CD56	5.7	13.6	nss	0.0	0.0	nss	8.4	5.8	nss	33.3	0.0	nss

*AML Acute myeloblastic leukemia

† B-ALL Acute lymphoblastic leukemia B cell

‡ T-ALL Acute lymphoblastic leukemia T cell

§ BAL Biphenotypic acute leukemia

3). CD45 was positive in 100% of the patients and CD34 in 2 (11.8 %) of them.

Cell marker expression in children with acute myeloid leukemia (AML)

Overall, 22 patients with AML were studied (mean: 8 years, median: 7.5 years, 95% CI=5.7-10.3 years). The cell markers with the highest frequency rate were: cMPO - 86.4 %, CD13 - 77.27%, CD33 - 72.72% and CD117 - 40.9%. CD34 marker was positive in 50% of the patients (Table 2).

Immunophenotypic diagnosis of AL in adults

After performing FCMI in 101 bone marrow and 96 peripheral blood samples (a total number of 197 adult patients), the immunophenotypic diagnosis were as follows: 139 (70.5%) patients were diagnosed with AML (mean age: 51.8 years, median: 54 years, 95% CI=49.0-54.7 years) and 53 (26.9%) with ALL (mean: 34.8 years, median age: 30.0 years, 95% CI=29.6-39.9). Three cases presented markers of two different cell lines and, hence, were classified as

biphenotypic acute leukemia (BAL). Two cases presented an undifferentiated immunophenotyping outcome because of the expression of only non-specific cell line markers CD45, CD34 and CD117 (Table 3).

Adult acute lymphoblastic leukemia (ALL)

In 53 (26.9%) patients with ALL, 41 (77.35%) of them resulted with lymphoid B cell line proliferation and 12 (22.65%) patients with a T cell line proliferation. In 41 ALL-B adults studied, the markers cCD79a and CD19 resulted with higher frequency rate (92.68%), followed by CD10 (70.7%) (Table 3). The CD45 marker was present with expressed intensity in all cases of ALL-T. The specific T cell marker CD7 was positive in 11 (91.6%) of the 12 patients studied. The intracellular T lymphoid cell marker cCD3 resulted positive in 83.33% of the patients, while the surface CD3, CD5, CD4, CD8 markers displayed the following frequency rates respectively: 50%, 83%, 58.33% and 41.66%. CD34 marker was positive in 4 (33.3%) of the 12

Table 3. General characteristics of the adult age patients studied, classified according to their AL subtype differentiation after MFCI

	B-ALL*	T-ALL [†]	AML [‡]	BAL [§]	UAL
Female sex No (%)	21 (51,2%)	5 (41,7%)	60 (43,2%)	1 (33,3%)	1 (50%)
Male sex No (%)	20 (48,8%)	7 (58,3%)	79 (56,8)	2 (66,6%)	1 (50%)
Mean age (years)	34.8	33.17	51.8	40.0	46.5
Median age (years)	30	31	54	-	-
95% CI (years)	29.6 - 39.9	22.2 - 44.1	48.9 - 54.7	-	-
Total No (%)	41 (20.8%)	12 (6,09)	139 (70,5%)	3 (1,5%)	2 (1,01%)

* B-ALL- Acute lymphoblastic leukemia B cell

[†] T-ALL- Acute lymphoblastic leukemia T cell

[‡] AML- Acute myeloblastic leukemia

[§] BAL-Biphenotypic acute leukemia

^{||} UAL-Undifferentiated acute leukemia

cases studied. The classical CD56 natural killer cell line marker resulted positive in one patient. The myeloid cell line marker CD33 was also found in one T-ALL patient (Table 2).

Intra and extra-cellular cell markers in adult patients with AML

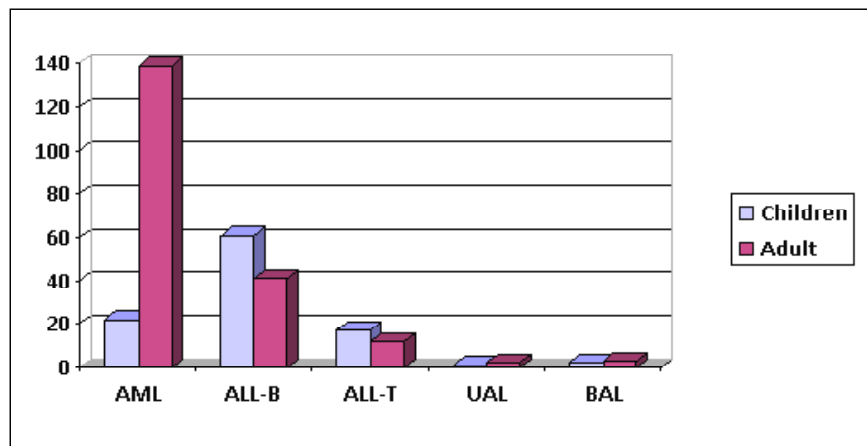
Overall, 139 adult patients resulted with AML diagnosis after FCMI. The specific myeloid cell line marker results ordered by the respective

frequency rate are presented in Table 2.

Comparison of AL subtypes in children and adultpatients

A higher frequency of ALL (75.7%) is observed in children compared to the adult patients (26.9%) (P=0.0001). AML was diagnosed in 70.5% of adult patients and in 21.35% of the children (P=0.0001). ALL-T diagnosis resulted in 16.5% of the children and in 6.09% of adult patients (Figure 2).

Figure 2. Comparison of AL subtypes after immunophenotypic diagnosis in adult patients and in children



Considering all the patients with ALL-B diagnosis, the CD45 marker positivity rate resulted in higher frequency rate in the adult patients group compared to the children group (P=0.045). On the

contrary, the CD10 marker was more frequently found among children than in adult patients (P=0.036). In the B-ALL children group with negative CD45 expression, the CD10 marker was

more frequently found compared to the ALL-B group of children expressing the CD45 marker ($P=0.0053$). The CD34 marker also displayed a higher frequency rate among the CD45 negative B-ALL children, compared to the counterpart CD45 positive B-ALL children ($P=0.017$).

Discussion

This study, based on the precise immunophenotypic definition of the affected cell line in patients suspected for AL, is the first research work enabling the refined classification of AL subtypes in Albania. Since the morphological diagnostic classification of AL is somewhat arbitrary, it is always recommended that the final diagnosis must be supported through MCFI (11,13). This is a crucial examination, since the precise definition of the immunophenotypic profile of the affected cell is indispensable in order to define the right treatment modalities and also to predict the prognosis of the disease (14).

Through the systematic use of the common leukocyte antigen marker CD45, combined with lineage-specific markers, it was possible to achieve a good discrimination between the abnormal leukemic cell population and the normal cells (15). This discrimination is based on the fact that the precursor cells in the bone marrow, as well as the leukemic cells, which derive from these cell types, express low to intermediate values of CD45 (16). The use of cell fixation/permeabilization technique for detecting not only surface expressed but also cytoplasmic and nuclear antigens, is a sensitive FCMI technique which enables the identification of cCD79a, cCD3, cMPO antigens as the earliest antigens appearing in the lymphoid and myeloid cell lines (17). This procedure was successfully implemented in our study, facilitating thus the accurate determination of the abnormal cell line subtype, since in some cases the immature blast cells lose their classical differentiation surface antigens (4,17). MFCI is also the only methodology that makes it possible to identify the biphenotypic acute leukemias (BAL) (18). We found in our

study five patients with BAL, of whom three patients expressed concomitantly abnormal B-cell and myeloid-cell markers, whereas two patients expressed T-cell and myeloid-cell markers. As also reported from other studies conducted in the European and American populations (19,20), ALL was found at a higher frequency rate in our study among the pediatric patients than in adults. Conversely, AML was found at a higher frequency rate (70.5%) among the AL patients of the adult age, a finding which is similar to other populations (21,22). The more sensitive and specific markers of this cell line were CD13 and CD33 which are present in all AML subtypes, whereas the intracellular MPO marker is expressed only in the M1, M2, M3 and M4 subtypes and is usually missing in the M0 and M5 subtypes (23).

MFCI provides a crucial help in order to classify the subtypes of AML. According to the EGIL criteria, the lack of HLA-DR, CD117 and CD34, together with the presence of CD13 and CD33 markers, strongly support the acute promyelocytic leukemia diagnosis, in a M3 type cyto-morphologically classified acute leukemia (24). We found in our study 15 patients who displayed such an immunophenotyping pattern, which helped to confirm this diagnosis. This is important in order to implement a treatment with ATRA (all-trans-retinoic acid) as a specific medication for this AML subtype (25). In the same way, the lack of cMPO in a myeloblastic cell population and the presence of CD14, CD64, CD4 cell markers in nine of our AML patients, supported the LAM-M5 diagnosis (monoblastic acute leukemia) in these patients (26). CD45 is an interesting prognostic marker in ALL (27,28). The lack of CD45, together with the presence of CD10, is a good prognostic marker in childhood ALL (29). We found this profile in 33.5% of our ALL pediatric patients. In contrast, we found the presence of CD45 in 82.9% of the adult patients with ALL, which could be an indicator of a poorer prognosis (30). The presence of the stem cell marker CD34 correlates also positively with the disease prognosis (31). We found this marker in 62.3% of our ALL pediatric patients and

in 51.2% of the adult patients putting in evidence the prognostic importance of these markers. Finally, we can conclude that the systematic implementation of FCMI plays a crucial role for the precise determination of the abnormal cells implicated and for the final diagnostic definition of AL subtypes of all Albanian patients regardless of

their age. FCMI helped enormously for the correct diagnosis of the AL cases with morphologically undifferentiated cells. Moreover, in three patients included in our study, the FCMI examination contributed to change the cyto-morphological diagnosis from one lineage to another, enabling thus the accurate AL diagnosis and the correct treatment.

Conflicts of interest: None declared.

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