Leukemia Research 107 (2021) 106603

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Lymphoid markers predict prognosis of pediatric and adolescent acute myeloid leukemia

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ARTICLE INFO

Keywords:
Acute myeloid leukemia
Prognosis
Immunophenotypic markers
CD7
CD22
CD4

ABSTRACT

Acute Myeloid Leukemia (AML) is a complex and highly aggressive disease. To characterize the prognostic factors of pediatric patients with AML relapse, a retrospective cohort study was performed to collect data from children and adolescents, at a hematological oncology reference center, over 11 years. We selected 51 cases of the disease, diagnosed and treated uniformly, divided into two groups: with complete remission (n = 33; 65 %) and with relapse (n = 18; 35 %). The groups were homogeneous concerning demographic characteristics and hematological parameters at diagnosis. AML M3 was the most common subtype (n = 19; 37 %) and was associated with a good prognosis. The highest rate of relapse was with AML M0 (n = 3 of 5 patients; 60 %). The most predominant gene mutation, FLT3-ITD, did not influence the prognosis in our study. The complete remission group presented a higher mean frequency of positive cells for the granulocytic marker CD13a at diagnosis. In cases with AML relapse, CD36, CD4, CD7, and CD22 were the most expressed markers. Increase incidence of recurrence was associated with CD7 (HR 1.035; p = 0.003), CD4 (HR 1.032, p = 0.001) and CD22 (HR 1.042; p = 0.049). Our results highlight the importance of analyzing immunophenotypic markers to help predict the outcome of AML in children and adolescents.

1. Introduction

One of the most common types of cancer in children and adolescents is leukemia, accounting for around one-third of all malignant diseases in this age group. Acute myeloid leukemia (AML), although not the most frequent, contributes to 15–20 % of childhood leukemia [1]. AML is an aggressive hematological disease that develops from cells of immature progenitors of myeloid origin, altering important processes of proliferation, self-renewal, and differentiation [2]. Childhood AML is characterized by important molecular and cytogenetic heterogeneity. The rapid progression and complexity of the disease require early diagnosis, and adequate and comprehensive treatment, together with the implementation of rehabilitation measures [3]. A challenging problem in the treatment of acute leukemia is related to disease recurrence, determined mainly by the biology of the disease and the emergence of resistant cell clones [4,5]. The AML recurrence rate in pediatric patients is approximately 30 %, despite initial remission; in addition, the death rate is high [6,7].

The prognosis for AML improved remarkably throughout the past decades, due to the optimization of existing treatment strategies [8]. Currently, a series of criteria that include chromosomal abnormalities, cytogenetics molecular markers, and clinical characteristics are used to identify patients with high or low risk of AML recurrence [9]. The immunophenotypic markers have proved to be an agile methodology for monitoring minimal residual disease in AML [10,11]. Analyses of surface and intracellular antigens using flow cytometry allow for differential diagnosis between lymphoid and myeloid leukemias, as well as for the distinguishing of lineage infidelity, the expression of abnormal antigens, and the presence of minimal residual disease. However, the prognostic value of immunophenotyping in AML remains controversial. On the one hand, some studies did not find any association with immunophenotyped markers and the survival of AML. On the other

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https://doi.org/10.1016/j.leukres.2021.106603
Received 4 February 2021; Received in revised form 24 April 2021; Accepted 28 April 2021
Available online 30 April 2021

hand, other studies have shown adverse prognosis regarding the expression of antigens such as CD7 [12,13], CD56 [14], and CD11b [15] and, more recently, CD34 [16] and CD318 [17]. These disparities between the studies and the lack of reproducibility are associated with different factors, such as technical constraints and analysis, and the impact of other prognostic factors. Also, few studies use immunophenotyping data as prognostic markers in pediatric patients. This study aims to use the lymphoid and myeloid markers in pediatric patients with AML to predict prognosis in a homogeneous population regarding treatment and diagnosis.

2. Patients and methods

2.1. Study population

A retrospective cohort study was conducted using medical information recorded from January 2008 to December 2019 at the Pediatric Hematology and Oncology service at the Hospital of the Medical School at Federal University of Santa Maria (HUSM), a regional reference center for pediatric cancer diagnosis and treatment, located in South Brazil. The study followed the REMARK guideline. Eligibility criteria included age up to 18 years, a medical diagnosis of AML, completion of treatment (or part of treatment undertaken) at HUSM, and follow-up of at least twelve months after diagnosis (until December 2019) to assess possible relapses.

2.2. Ethics

The Human Research Ethics Committees of the Federal University of Santa Maria (nº 047032) and the Pontifical Catholic University of Rio Grande do Sul (CAAE nº 86206218.7.1001.5336) approved the study protocol.

2.3. AML diagnosis

AML diagnosis was based on analysis of bone marrow immunophenotyping (CD45, HLA-DR, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD13, CD14, CD15, CD19, CD20, CD22, CD33, CD34, CD38, CD64, CD117, cCD3, cCD79a, cMPO, Terminal desoxinucleotidil Transferase (TdT)), molecular markers (PML-RARA, RUNX1-RUNX1T1, CBFB-MYH11, FLT3-ITD, BCR-ABL p210 e p190 e MLL-AF4), and chromosomal abnormalities such as t(15;17), t(8,21), t(3,5), del(9), add(1), inv(16), hypodiploidy, hyperdiploidy, and Down syndrome. The immunophenotyping was performed in bone marrow after incubation with lyse wash buffer and staining with antibodies from BD Bioscience, Caltag, Beckman Coulter, Dako and Exbio. The analysis was performed in the flow cytometer FACS Calibur (Becton Dickinson) and the software Cell Quest Pro was used to data analysis.

The AML types were established, according to the criteria of the French-American-British (FAB) classification system, at M0, M1, M2, M3 (Acute Promyelocytic Leukemia), M4, M5, M6, and M7.

2.4. Independent variables

Patient baseline characteristics included sex (male, female), age (1−10, 11−15, >15 years), ethnicity (Caucasian, no Caucasian), clinical symptoms (bleeding, pain, fever, gastrointestinal symptoms, asthenia, bruises, headache, lymphadenomegaly, pallor, cough, and inappetence), hematological parameters (white blood cell count in 10^3/µl, hemoglobin level in g/dL, plaquette count in 10^3/µl, and % blats), and type of treatment.

2.5. Statistical analyses

Data were analyzed using the SPSS 19.0 statistical program. Hematological parameters were presented as medians with interquartile intervals (IQ). Student's *t*-test was used to compare variables with symmetric distribution and Fischer Exact and Mann-Whitney non-parametric tests were used to compare variables with asymmetric distribution. For all tests, the minimum significance level of 5% ($p < 0.05$) was considered. The survival analysis was estimated by Survival Kaplan-Meier curve and non-parametric log-rank test (Mantel-Cox) or hazard ratio (HR). The HR calculation was provided using the proportional hazards model (COX regression), using a confidence interval of 95 %.

2.6. Ethics

The Human Research Ethics Committees of the Federal University of Santa Maria (nº 047032) and the Pontifical Catholic University of Rio Grande do Sul (CAAE nº 86206218.7.1001.5336) approved the study protocol.

2.7. Outcomes

Complete remission (CR) was defined as the absence of disease signs and symptoms and of leukemic blasts, and recovery of normal hematopoietic cells in the bone marrow after receipt of standard induction treatment. Relapse was defined as the return of disease activity, as ascertained by the reappearance of leukemic blasts in peripheral blood or bone marrow, equal to or greater than 5% (in the absence of extra causes, such as medullary recovery after chemotherapy). Refractory AML was defined for children and adolescents who, after receiving two induction cycles of treatment, did not reach CR.

3. Results

All 1,061 medical records from January 2008 to December 2019 of the Pediatric Hematology-Oncology unit were screened. Of these, 345 were pediatric leukemia cases, 280 of which were cases of acute lymphoblastic leukemia (ALL) and 65 of AML. After application of the eligibility criteria, 51 children and adolescents with AML were enrolled in the study. The general average age was 11.4 (standard deviation = 5.1) years, ranging from 1.3 to 18.8 years. A total of 27 children and adolescents were male, and most of them were Caucasian (47/51).

In most of the cases (n = 29; 57 %), the treatment schedule followed the Brazilian protocol adapted from AML-IO 97 (chemotherapy cycle composed of Daunorubicin, Ara-C, and Vepesid in the induction phase, with replacement of the anthracycline Daunorubicin by Mitoxantrone in the consolidation phase) [18]. Other protocols were adapted for specific cytogenetic changes in 6% of the patients (n = 3). Pediatric patients with AML-M3 (n = 19; 37 %) were treated with all-trans-retinoic acid (ATRA) or arsenic trioxide (ATO) associated with the administration of a chemotherapy protocol.

Of the 51 cases of AML, 33 (65 %) achieved CR of the disease and 13 (25 %) had disease recurrence. In all cases, relapses occurred in the bone marrow. Only two cases had the second relapse in the central nervous system. Seventeen patients (33 %) died in the course of the disease: four cases with CR and 13 with relapse or refractory disease. Four of them (23 %) were due to the progression of AML, while the remaining were consequences of treatment-related causes: septicemia (n = 6; 35 %), respiratory failure (n = 2; 12 %), acute renal failure (n = 2; 12 %), cardiogenic shock (n = 1; 6%), ATRA syndrome (n = 1; 6%), and tumor lysis syndrome (n = 1; 6%). The global median survival time was six years, and the median event-free survival time was 2.6 years (Fig. 1).

There were no differences between children and adolescents with CR and those who relapsed or recurrence in terms of demographic characteristics, symptoms, or hematological parameters at diagnosis (Table 1). The most frequent symptoms reported at diagnosis by children and adolescents with or without CR were bleeding, fever, and asthenia (Table 1). The most frequent type of AML was subtype M3, accounting for 19 cases (three of whom had disease relapse). The relapse rate of 16

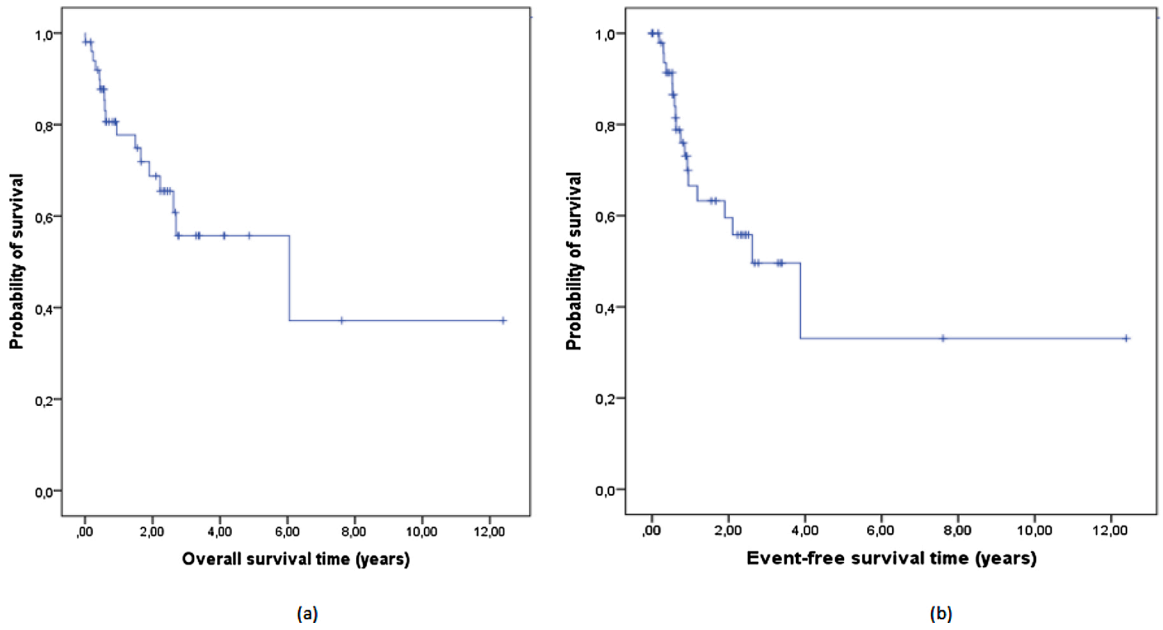

Fig. 1. Kapplan-Meier for overall and event-free survival time to all AML children and adolescent patients.

% (3/19) in AML M3 cases was low as compared to the other types: M0 - 60% (3/5), M1 - 29% (2/7), M2 - 45% (5/11), M4 - 40% (2/5), M5 - 67% (2/3), and M6 - 100% (1/1) (Table 1). None of the patients were affected by the M7 subtype.

Molecular and cytogenetic markers observed in children and adolescents under study are summarized in Table 2. The FLT3-ITD gene mutation was detected in 10 patients, six of whom presented CR (Table 2). There were no difference in global ($p = 0.161$) or event-free survival time curves ($p = 0.549$) between patients with or without the presence of FLT3-ITD gene mutation (Fig. 2A). However, when a subgroup analysis was performed with the non-M3 patients the presence of FLT3-ITD was significant related with less overall survival time ($p = 0.004$) and less event-free survival time ($p = 0.001$) (Fig. 2B)

We evaluated the antigen expression profile determined during immunophenotyping at diagnosis among children and adolescents with AML and compared patients with complete remission to those who relapsed. We used the mean frequency of positive cells to perform this analysis. The median frequency of CD13 positive cells at diagnosis was higher in patients with CR as compared to those who relapsed (Table 3). In contrast, the frequency of CD36, CD7, CD22, and CD4 was significantly lower at diagnosis in patients with CR as compared to those who relapsed (Table 3). There was no statistical difference between patients with CR or relapse when comparing the percentage of positive cells for the precursor markers CD45, CD34, and CD117; monocytic markers CD64; or granulocytic markers CD15, CD33, and MPO. Similarly, the median frequency of aberrant phenotypes tested—CD19, CD2, and CD56—were not different between groups (Table 3). We performed a subgroup analysis with only the non-M3 AML patients and the median frequency of CD13 positive cells continued higher in patients with complete remission compared to those who relapse (Table 4). Also, the median frequency of the cells positive for the lymphoid marker CD22 continued to be significantly lower in patients with complete remission (Table 4).

We further analyzed the Hazard ratio for association with AML recurrence using demographic data and clinical parameters. As expected, the subtype AML M3 is associated with a better prognosis (Fig. 3). Although we found a tendency for an association of FLT3-ITD with decreased incidence of recurrence this was not statically significant in our analysis (Fig. 2). The markers CD22, CD7, and CD4 were associated with an increased incidence of recurrence (Fig. 2).

4. Discussion

The main findings of our study are that 1) AML M3 was the most frequent subtype of AML in the studied population; 2) 35 % of the children and adolescents with AML relapsed within one year after diagnosis; 3) the relapse rate in AML M3 cases was lower than in the other subtypes of AML; 4) the percentage of cells positive for CD13 at diagnosis was higher among patients who evolved to CR; 5) the percentage of CD36 positive cells at diagnosis was higher in patients who had a recurrence of the disease; and 6) the higher frequency of markers of anomalous expression of CD4, CD7, and CD22 at diagnosis was associated with subsequent recurrence of the disease.

The 35 % relapse rate observed in our study is similar to findings of other studies performed in Brazil [19,20]. In the present cohort, the recurrence was mainly in bone marrow, in agreement with De Lima et al. [21], who observed bone marrow recurrence in 14 % of AML cases.

In our study, relapse of AML in children and adolescents was not related to any demographic data, clinical manifestations, or hematological analysis performed in peripheral blood at diagnosis. The predominance of males, Caucasian ethnicity, and those aged between 1 and 10 years old in our sample corroborates other studies [21,22]. Clinical symptoms and signs at diagnosis were similar to what the literature has described, with nonspecific manifestations such as fever, vomiting, weight loss, bleeding, generalized adenomegaly, generalized bone pain, and pallor, which can be present in many common childhood diseases [23–25]. Anemia and thrombocytopenia were observed in both cases with CR or relapse. The presence of leukocytosis, with the IQ interval within 10,000 and 50,000 cells/μl in the whole cohort, is similar to what has been previously observed in other studies [22,25,26].

Our data showed a higher prevalence of AML M3 cases. Over one-third (37 %) of all AML cases were AML M3, which was similar to an epidemiologic study also conducted in the south region of the country, which found 29 % of AML M3 cases [26]. The frequency of M3 among AMLs is highly variable, with a predominant incidence in countries with 'Latin' colonization [27,28]. For instance, in the pediatric population of the northern region of Brazil, surveyed in 2015, the most frequent AML types were AML M2 (38 %), followed by AML M0 and LMA-M1 (both 28 %) [29]. This pediatric population presented a low frequency of AML M3 (10 %) [29]. Such findings are similar to the results of studies in most developed countries, in which the prevalence rate of AML M3 varies

Table 1
Frequency of complete remission (yes, no) according to demographic characteristics, clinical symptoms and hematological parameters and FAB classification at diagnosis among pediatric and adolescent patients with Acute Myeloid Leukemia.

Characteristics	Complete remission		p
	Yes (n = 33)	No (n = 18)	
Sex			0.756[1]
Female	15 (62 %)	9 (38 %)	
Male	18 (67 %)	9 (33 %)	
Ethnicity			0.443[2]
Caucasian	31 (66 %)	16 (34 %)	
No Caucasian	2 (50 %)	2 (50)	
Age (years)			0.637[1]
1–10	17 (71 %)	7 (29 %)	
11–15	7 (64 %)	4 (36 %)	
> 15	7 (44 %)	9 (56 %)	
Clinical symptoms			
Bleeding	18 (67 %)	9 (33 %)	0.756[1]
Pain	13 (62 %)	8 (38 %)	0.726[1]
Fever	15 (62 %)	9 (38 %)	0.756[1]
Gastrointestinal symptoms	6 (46 %)	7 (54 %)	0.101[2]
Asthenia	14 (58 %)	10 (42 %)	0.120[1]
Bruises	9 (90 %)	1 (10 %)	0.062[2]
Headache	3 (38 %)	5 (62 %)	0.091[2]
Lymphadenomegaly	8 (73 %)	3 (27 %)	0.401[2]
Pallor	3 (75 %)	1 (25 %)	0.557[2]
Cough	2 (67 %)	1 (33 %)	0.718[2]
Inappetence	5 (62 %)	3 (38 %)	0.591[2]
Hematological parameters			
Leucocytes (x 10^3/µl), median (IQ[3])	11.20 (3.55–29.90)	23.01 (9.93–66.60)	0.129[4]
Hemoglobin, g/dL, median (IQ[3])	7.80 (6.25–8.95)	6.65 (4.77–9.12)	0.261[4]
Plaquettes (x 10^3/µl), median (IQ[3])	21.00 (13.00–45.50)	34.50 (17.50–62.75)	0.161[4]
Blasts (%), median (IQ[3])	70.00 (26.00–83.50)	31.50 (19.50–83.25)	0.484[4]
FAB classification			
Mo	2	3	
M1	5	2	
M2	6	5	
M3	16	3	
M4	3	2	
M5	1	2	

[1] Chi-square test.
[2] Fischer Exact test.
[3] IQ: interquartile interval.
[4] Mann-Whitney test.

from 4% to 15 % [30].

In our study, the relapse rate of in AML M3 cases was low as compared to the other types. The effectiveness of the management of AML M3 improved with the use of molecular targeted therapy, such as the incorporation of all-trans-retinoic acid (ATRA) and arsenic trioxide (ATO) in the conventional therapeutic regimen. These new therapy strategies present low toxicity and reduce the incidence of complications due to infections [31,32].

Previous studies to characterize the FLT3-ITD mutation [33] and analyze the prevalence and clinical significance of the FLT3 mutation in patients with AML [34] concluded that the FLT3 mutation is associated with a worse prognosis. Meshinchi et al. [35] observed that disease remission in the induction period was 40 % in patients with positive FLT3-ITD, compared to 74 % in patients with negative FLT3-ITD. Several authors have found unfavorable results for induction chemotherapy, worse prognosis, and a high risk of relapse in FLT3-ITD patients [20,35,36]. In our research, an unfavorable influence was not identified in patients with the FLT3-ITD mutation associated with AML. However, when we analyzed only the non-M3 AML we found that FLT3-ITD mutation is associated with poor outcome.

Regarding the influence of the antigen expression profile, children and adolescents who achieved CR presented an increased percentage of

Table 2
Frequency of chromosomal alteration and molecular markers in children and adolescents with AML.

	Molecular Biology Markers	n	Complete Remission	
			yes n(%)	no n (%)
del(11)(q23)		1	1 (100)	0 (0.0)
add(1)(p36)		1	0 (0.0)	1 (100)
Hyperdiploidy		1	0 (0.0)	1 (100)
Hypodiploidia		2	2 (100)	0 (0.0)
t(8;21)	RUNX1-RUNX1T1	7	5 (71.4)	2 (28.6)
inv(16)	CBFB-MYH11	4	3 (75.0)	1 (25.0)
Normal	FLT3-ITD (+)	6	0 (0.0)	6 (100)
Normal	FLT3-ITD (-)	5	2 (40.0)	3 (60.0)
t(15;17)	PML-RARA	10	8 (80.0)	2 (20.0)
t(15;17)	PML-RARA/FLT3-ITD	6	6 (100)	0 (0.0)
t(15;17)	NR	3	3 (100)	0 (0.0)
NR	FLT3-ITD (+)	1	1 (100)	0 (0.0)
Down's syndrome		1	1 (0.0)	0 (100)
t(3;5)(q25;q34)		1	1 (0.0)	0 (100)
t(9;11)		1	1 (100)	0 (0.0)
del(5)(q12q33)		1	1 (0.0)	0 (100)
Total		51	35 (68.6)	16 (31,4)

NR = exam not performed; + presence of genetic alteration; - absence of genetic alteration.

cells positive for CD13 at diagnosis, suggesting a favorable association. Also, the association with CD13 and complete remission remains when analyzing only the non-M3 AML patients. Divergent data showed a significant association between the presence of the CD13 marker and cases of relapse, but this study was performed in adults [37].

The higher frequency of CD36 positive cells at diagnosis was identified in cases that presented recurrence of the disease. This result coincides with the findings of other studies, which suggested a reduction in the leukemia-free survival rate to two years and a shorter overall survival time in the AML in adult patients who presented a high frequency of CD36 positive cells at diagnosis [38]. Controversial results have been described when comparing the patterns of immunophenotypic and drug sensitivity in cases of acute megacarioblast leukemia in childhood, in which an increase in sensitivity to chemotherapy drugs was identified when the blasts had high CD36 expression, suggesting a better prognosis of the disease [39]. This disagreement occurs because the prognostic role of immunophenotyping in AML has still been conflicting in many cases.

Research carried out in Iraq by Bhushan et al. [40] concluded that lymphoid phenotypes were present in different leukemia subtypes. The CD7 marker was one of the lymphoid antigens repeatedly expressed in cells of patients with AML [41,42]. The expression of aberrant CD19 and CD7 phenotypes is more prevalent in children and adolescents than in adults [40]. Higher frequency of the markers of the anomalous expression of CD4, CD7, and CD22 at diagnosis was associated with subsequent recurrence in children and adolescents with AML in our study. Interestingly, we also found that the CD22 expression is associated with relapse in the non-M3 AML patients. CD22 is a target for ALL treatment. In agreement with our results, Rodríguez-Rodríguez et al. [43], in a survey conducted with 433 patients affected by AML, observed that the presence of lymphoid markers CD7, CD19, CD2, and CD22 was associated with unfavorable clinical outcomes.

Our study has strengths and limitations. Among the strengths is the use of a longitudinal study design. All AML cases in children and adolescents diagnosed and treated over the course of 11 years, in a public institution of reference for pediatric cancer treatment in Brazil, were followed up for prognosis purposes. All the cohort participants were diagnosed and treated uniformly throughout that time. The incidence of AML is low. In Brazil, the estimated incidence is at around 400 annual cases. Thus, the number of cases investigated in this study is expressive of a single reference center [44]. However, the findings of our study must be interpreted with caution due to the small sample size.

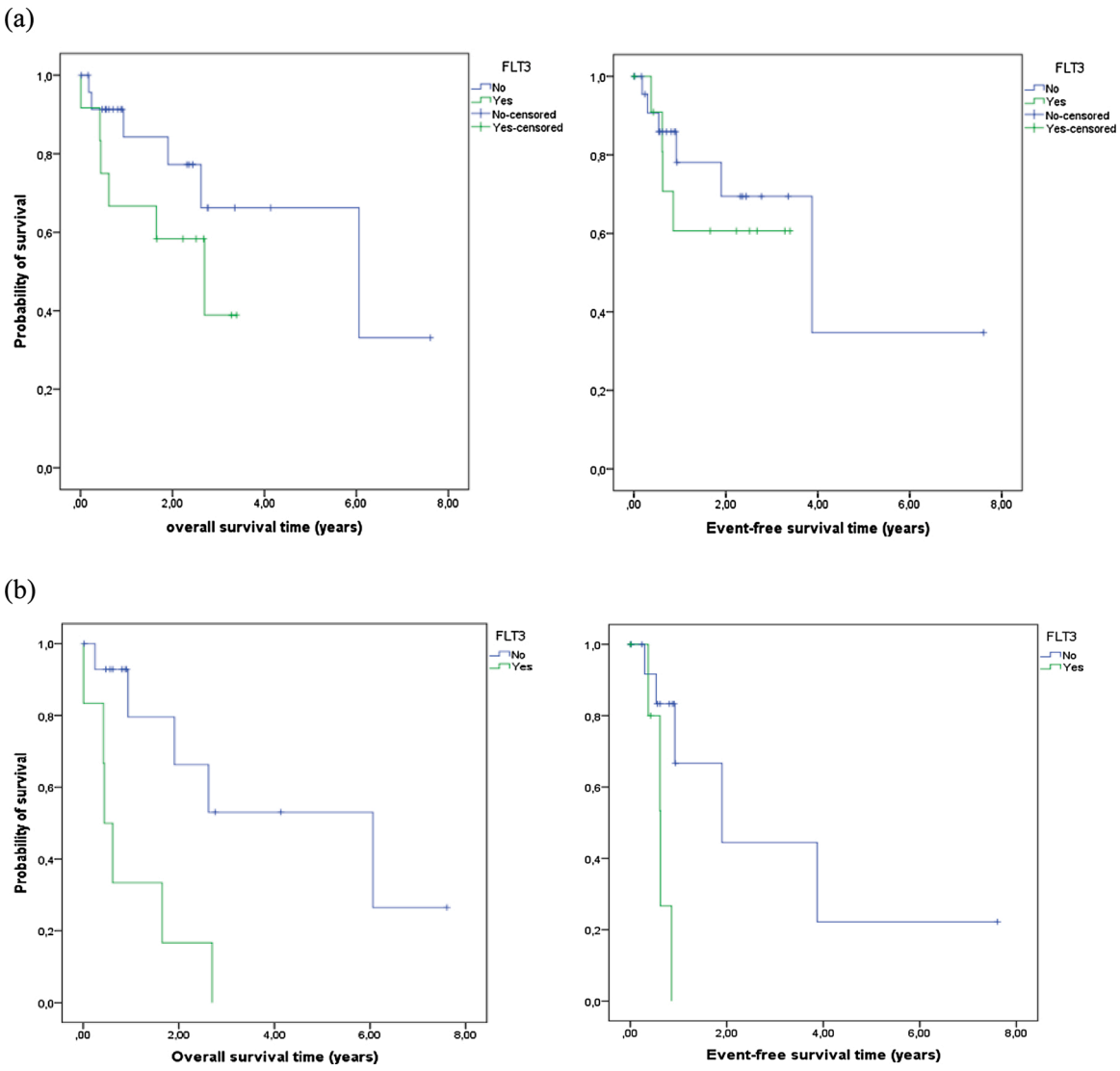

Fig. 2. Kaplan-Meier for overall and event-free survival time to all AML (a) and non-M3 AML children and adolescent patients.

Table 3
Median of the percentage of positive cells for immunophenotyping markers among pediatric and adolescent patients with Acute Myeloid Leukemia with or without complete remission.

Complete Remission	Yes		No		
	n	Median (IQ)	n	Median (IQ)	p
CD13	33	82 (67.5–90.5)	18	62 (43.75–82.75)	0.020*
CD36	10	0 (0–11.25)	9	32 (9.5–52)	0.007*
CD22	26	1 (0.75–3.25)	14	5.5 (1.5–24.5)	0.008*
CD7	27	3 (1–8)	13	30 (4.5–57)	0.006*
CD4	30	2 (1–4.2)	15	8 (3–40)	0.004*
CD45	33	73 (59–80)	18	57.3 (46–74)	0.061
CD34	32	19 (3–45.5)	17	54 (7–70)	0.162
CD117	28	71.5 (40.75–78)	16	52(44.5–72.75)	0.213
CD64	26	36 (9.5–71.75)	12	70.5 (9.5–82.25)	0.550
CD15	29	4 (1–18)	15	2 (0–8)	0.319
CD33	31	90 (79–94)	16	91 (82.25–97.75)	0.543
MPO	18	85 (46.50–96)	10	81.5 (35–92.25)	0.579
CD19	32	2 (1–15.5)	18	5.5 (1–23)	0.216
CD2	28	2.5 (1–9.7)	16	1.5 (0–4.7)	0.182
CD56	29	3 (0–15.5)	11	4 (1–36)	0.377

* $p < 0.05$ Mann-Whitney test, IQ (interquartile interval).

Table 4
Median of the percentage of positive cells for immunophenotyping markers among pediatric and adolescent patients with non-M3 AML with or without complete remission.

Complete Remission	Yes		No		
	n	Median (IQ)	n	Median (IQ)	p
CD13	17	71 (62.5–89.0)	15	61 (40.0–79.0)	0.030*
CD36	7	0 (0–36)	9	9.5 (4–32)	0.064
CD22	13	2 (0.5–4.0)	12	6.0 (3.5–29.5)	0.006*
CD7	15	5 (3–11)	11	30 (6–62)	0.099
CD4	15	3 (1–29)	14	10 (3.75–43.5)	0.174
CD45	17	69 (51.5–80)	15	55.0 (40–64)	0.080
CD34	15	43 (14.0–78.0)	15	54 (10–69)	0.172
CD117	14	68.5 (32.2–74)	13	52 (47–70.5)	0.557
CD64	14	22 (6.7–81.0)	11	73 (6–85)	0.599
CD15	13	11 (1–52)	13	3 (0–11.5)	0.338
CD33	15	89 (83–92)	13	89 (76–97)	0.954
MPO	10	66.5 (22.7–86)	10	81.5 (35–92.25)	0.593
CD19	17	2 (1–5.5)	15	6 (1–38)	0.126
CD2	14	2.5 (0.75–6.25)	13	2.0 (0.5–5.5)	0.620
CD56	15	2 (0–41)	10	5 (1–40)	0.611

* $p < 0.05$ Mann-Whitney test, IQ (interquartile interval).

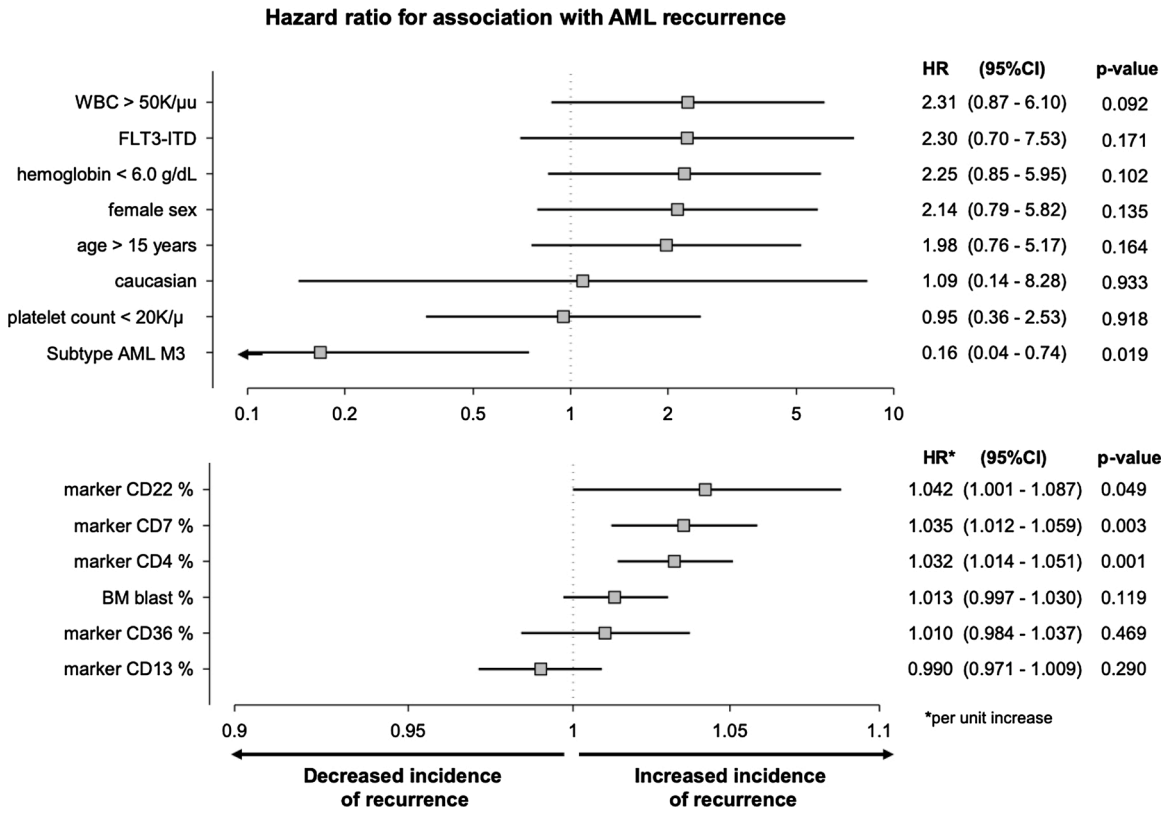

Fig. 3. Hazard ratio analysis in children and adolescents with AML using different clinical parameters and immunophenotyping markers.

In conclusion, our study showed a better prognosis for patients with the subtype AML M3. A worse prognosis was observed when the percentage of positive cells for lymphoid markers CD4, CD7, and CD22. Our results highlight the importance of analyzing immunophenotypic markers to help predict the outcome of AML in children and adolescents.

Funding

This study was supported in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Finance Code 001.

Ethics approval

The Human Research Ethics Committees of the Federal University of Santa Maria (nº 047032) and the Pontifical Catholic University of Rio Grande do Sul (CAAE nº 86206218.7.1001.5336) approved the study protocol.

Author's contributions

Rosmeri Hoch and Ana Paula Duarte de Souza contributed to the study conception and design. Material preparation and data collection were performed by Rosmery Hoch. Data analysis were performed by all authors. All authors reviewed the manuscript and approved the final version.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgment

The authors thank the registration team of HUSM.

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