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Original article

Influence of minimal residual disease by multiparametric flow cytometry at day 15 of induction in risk stratification of children with B-cell acute lymphoblastic leukemia treated at a referral hospital in southern Brazil

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ABSTRACT

Background: The minimal residual disease (MRD) is the most important prognostic factor for acute lymphoblastic leukemia (ALL) in children. This study aimed to investigate the influence of detecting the MRD by the multiparametric flow cytometry (MFC) at day 15 (D15) of the induction on the analysis of the risk group classifications of the different childhood ALL treatment protocols used in a referral hospital in southern Brazil.
Method: We retrospectively reviewed the medical records of patients with B-cell ALL, aged 1 to 18 years, treated at a hospital from January 2013 to April 2017.
Main results: Seventy-five patients were analyzed. Regarding the MRD by the MFC at D15, the analyses showed statistical significance when the MRD was grouped into three categories, < 0.1%, 0.1–10%, and > 10%, with the following distribution: 30.7%, 52.0%, and 17.3%, respectively. There was a significant association between D15 MRD-MFC < 0.1% and the likelihood of dying or relapsing and between D15 MRD-MFC > 10% and the likelihood of dying or relapsing. The cumulative hazard ratio for the relapse of patients with D15 MRD-MFC < 0.1%, 0.1–10%, and > 10% was 19.2%, 59.8%, and 80.1%, respectively.
Conclusion: Our analysis suggests D15 MRD-MFC < 0.1% as a cut-off point for patients with more favorable outcomes and that the MRD at D15 in risk classifications is particularly useful for the stratification of patients with a more favorable prognosis.

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Introduction

Childhood acute lymphoblastic leukemia (ALL) is now a potentially curable disease in most cases. Advances in science have allowed overall survival rates, which were less than 10% in 1960, to rise to approximately 90% in the United States.[1] Despite this significant improvement, approximately 20% of the patients still relapse, with a cure rate after relapse of approximately 25–40%.[2,3]

Relapse is the leading cause of treatment failure. Early response to therapy is an important prognostic indicator and may predict this risk.[4] Patients with rapid clearance of blasts in peripheral blood after 1 week of corticosteroid therapy usually have a more favorable prognosis.[5] The same is true for patients with a reduction in bone marrow blasts at day 15 (D15) of the induction.[6]

Most study groups currently explore the measurement of the kinetics of leukemic cells at the submicroscopic level, that is, the minimal residual disease (MRD). The MRD is the most important prognostic factor for ALL in children and has been used to refine risk stratification in most modern treatment protocols and to redirect treatment.[7]

Molecular detection of the MRD has been well standardized. Polymerase chain reaction (PCR) techniques are more sensitive and can detect one leukemic cell in 100,000 normal cells. Traditionally, the detection limit of multiparametric flow cytometry (MFC) is 0.01% or 10^{-4}, that is, the MFC can detect one leukemic cell in 10,000 normal cells.[8] However, next-generation flow MRD has a sensitivity similar to that of immunoglobulin (Ig) and T-cell receptor (TCR) rearrangements, which is currently considered the gold standard for MRD detection.

Detection of the MRD, especially by molecular biology, at the end of the induction has been used by almost all study groups as a determinant of treatment intensity after the induction therapy. The clinical significance of the MRD, at very low levels or undetectable disease, is well established as an indicator of a more favorable outcome and lower risk of relapse.[9]

The Associazione Italiana di Ematologia Oncologia Pediatrica and the Berlin-Frankfurt-Münster ALL (AIEOP-BFM ALL) 2000 study, however, showed that the bone marrow measurement of the MRD by the MFC on D15 also has a strong prognostic impact and may complement risk stratification.[10]

The present study aimed to investigate the influence of detecting the MRD by the MFC at D15 of the induction in the analysis of risk group classifications in patients classified according to the different childhood ALL treatment protocols used in a referral hospital in southern Brazil.

Methods

Study population

We conducted a descriptive cross-sectional epidemiological study with retrospective data collection from 75 patients, aged 1–18 years, with B-cell ALL (B-ALL) treated at a hospital from January 2013 to April 2017. Exclusion criteria were missing data for the analysis of the MRD at D15 of the induction or non-B-cell ALL. The bone marrow aspirate was examined at D15 (after 14 days of corticosteroids, 1 dose of vincristine, daunorubicin and asparaginase, and 1 methotrexate intrathecal) and at the end of induction for morphological evaluation and MRD studies.

Flow cytometric immunophenotyping of bone marrow or peripheral blood was performed in all cases at diagnosis. The patient medical records were reviewed for data on age, leukocyte count, central nervous system (CNS) involvement, morphology of leukemic cells, cytogenetics and molecular biology at diagnosis.

The MRD was assessed by immunophenotyping with a BD FACSCantoII flow cytometer (Becton Dickinson, San Jose, CA, USA) using a 6-color MFC until the end of 2015 and an 8-color MFC thereafter. The cell labeling protocols were based on Euroflow standardization.[11]

Briefly, for the surface antigens, 100 μL fresh samples were incubated with different amounts of monoclonal antibodies, as obtained from titration labeled antibodies for 15 min at room temperature. The cells were then lysed according to the manufacturer instructions (BD FACS Lysing Solution; BD Biosciences, San Jose, CA, USA), incubated for 10 min at room temperature in darkness and centrifuged (5 min. at 540*g*). After another centrifugation step (5 min. at 540*g*), the supernatant was discarded and the cell pellet was resuspended in 250 μl of phosphate-buffered solution (PBS) for analysis. For the detection of intracellular antigens, a FIX&PERM® Cell Permeabilization Kit (Invitrogen™, Carlsbad, CA, USA) was used. After the first incubation with conjugated surface antibodies for 15 min, 100 μL of Fix&Perm reagent "A" was added for 10 min. After being washed once, the cells were permeabilized by the addition of 100 μL of Fix&Perm reagent "B" and the unconjugated intracellular antibodies were added for a 15-minute incubation. After another centrifugation step (5 min. at 540*g*) and wash, the cells were resuspended in phosphate-buffered saline (PBS) for analysis.

For the MRD assessment, we used two tubes with the antibodies CD19, CD45, CD34, CD10, CD38, CD20 and CD81 distributed in 6 colors (FITC, PE, PerCP, PE-CY7, APC, APC-H7), and the antibodies CD66c and CD123 were used with the addition of 8 colors (Pacific Orange and Pacific Blue). For CD10 negative samples, a combination of CD65, CD15 and NG2 was used in the panel, as it is associated with (11q23) KMT2A (MLL), whose phenotype is $CD10^{-}/CD15^{+}/CD65^{+}/7.1(NG2)^{+}$,[12,13] as well as the phenotype $CD66c^{+}/CD34^{+}/CD10^{+}/CD38^{-/weak}$, which suggests t(9;22)(q34;q11):BCR/ABL1.[12,14] For accurate detection of low frequencies of malignant cells, that is, 1 in 10,000 normal cells (0.01% or 10^{-4}), a minimum of 1,000,000 events were acquired. The MRD monitoring was performed by detecting normal vs. reactive vs. clonal immunophenotypes. The MRD detection strategies are illustrated in Figure 1. All data were analyzed using the Infinicyt™ (Cytognos, Salamanca, Spain).

The study was approved by the Hospital Research Ethics Committee (approval number: CAAE 55303916.5.0000.5327).

Definition of risk groups

Risk stratification was based on the National Cancer Institute (NCI) criteria and on the criteria of the following

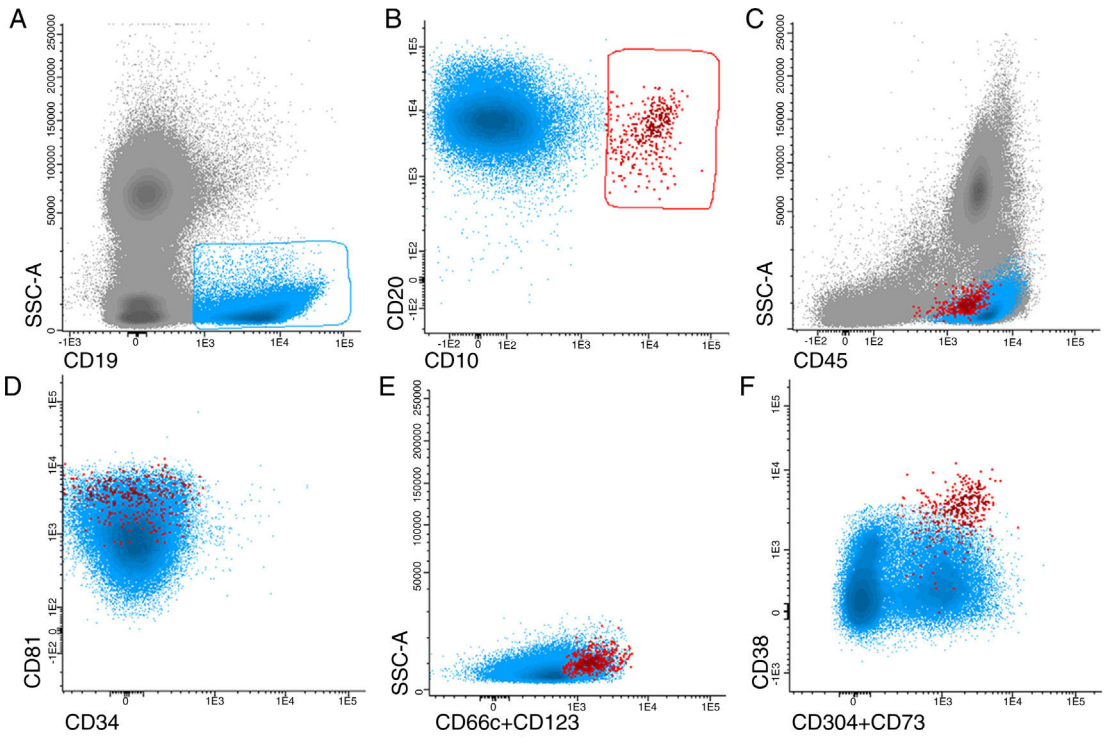

Figure 1 – Positive minimal residual disease (MRD) with gate strategies. (A) Gate in B lymphoid cells (CD19 +); (B) CD10 + clonal cell population (in red) with CD20 expression (blue population - mature B lymphoid cells); (C) Position of clonal and mature cells on the CD45 chart; (D) CD81-expressing clonal cells without CD34-expression; (E) Clonal cell positivity for CD66c/CD123; and (F) Clonal cell positivity for CD304/CD73 and CD38.

Berlin-Frankfurt-Münster (BFM) group protocols: BFM 95; Intercontinental (IC)-BFM 2002; and IC-BFM 2009.[15–18] All patients were stratified according to their clinical and laboratory characteristics at diagnosis and speed of response to initial treatment regardless of the treatment protocol used.

The risk classification system of the IC-BFM 2009 protocol, which includes the MRD for risk stratification, was compared with the risk classification systems of previous protocols that do not include the MRD.

Data structure and statistical analysis

Data were entered onto an Excel spreadsheet, version 2016 and then exported to SPSS, version 20.0, for statistical analysis. Survival curves were estimated by the Kaplan-Meier method. The mean survival time was compared by the log-rank test. The cumulative risk of relapse or death was expressed as hazard ratio (HR). Overall survival was measured from the date of the diagnosis of ALL to the date of death or last contact. Event-free survival was measured from the date of the diagnosis of ALL to the date of relapse, refractoriness to treatment, death, or last contact. All data were updated to October 2017, and a p-value < 0.05 was considered significant.

Results

A total of 75 patients, aged 1–18 years, with a diagnosis of B-ALL were analyzed. Of the 75 patients, 16 (21.3%) relapsed and 17 (22.6%) died.

The distribution of the risk groups, by comparing the risk classification of the IC-BFM 2009 protocol with the risk classifications of the BFM 95 and IC-BFM 2002 protocols, is shown in Table 1.

Initially, the MRD at D15 and the MRD at the end of the induction were divided into four categories: <0.01%; 0.01–0.1%; 0.1–10%; and >10%.

Regarding the MRD at the end of the induction, there was a significant association between MRD 0.1–10% and relapse ($p = 0.001$; chi-square test) and between the MRD >10% and death and relapse ($p < 0.001$; chi-square test). All patients with the MRD >10% at the end of the induction died. Conversely, patients with the MRD <0.01% were less likely to die or relapse ($p < 0.001$; chi-square test) (Table 2).

Regarding the MRD at D15, the survival curves divided into four categories (<0.01%, 0.01–0.1%, 0.1–10%, and >10%) were neither significant nor representative. The curves showed significant results to demonstrate the groups at higher and lower risk of relapse or death only when values were grouped into three (rather than four) categories: <0.1%; 0.1–10%; and >10%. Clinically, the results suggest a cut-off D15 MRD-MFC value of 0.1% for low risk, and of >10% for high risk, of relapse or death. There was a significant association between D15 MRD-MFC <0.1% and a lower likelihood of death ($p = 0.006$; chi-square test) or relapse ($p = 0.03$; chi-square test) and between D15 MRD-MFC >10% and a greater likelihood of death ($p = 0.006$; chi-square test) or relapse ($p = 0.03$; chi-square test) (Table 3).

Table 1 – Agreement analysis of risk classifications between IC-BFM 2009 and BFM 95 protocols and between IC-BFM 2009 and IC-BFM 2002 protocols.

Protocols			IC-BFM 2009 RISK			Total
		LR	IR	HR		
	BFM 95 risk	LR	8	13	2	23
	IR	0	29	1	30	
	HR	0	0	22	22	
Total			8	42	25	75
IC-BFM 2002	LR		8	12	1	21
Risk	IR		0	30	2	32
	HR		0	0	22	22
Total			8	42	25	75

LR: low-risk; IR: intermediate-risk; HR: high-risk. BFM: Berlin-Frankfurt-Münster group; IC: Intercontinental.

Table 2 – Distribution of events according to MRD at end of induction by MFC.

Events	<0.01%		0.01–0.1%		0.1–10%		>10%		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
Total	63	84	4	5.3	4	5.3	4	5.3	75	100
Relapse	9		1		4		2		16	21.3
BM	5		1		2		1			
Testis	1			1						
CNS	2									
BM + CNS	1				1					
BM + lymph nodes			1							
Deaths	10		1		2		4		17	22.6
Disease			2		3					
In CR after CT	3		1							
In CR after BMT	7				1					

MRD: minimal residual disease; MFC: multiparametric flow cytometry; BM: bone marrow; CNS: central nervous system; CR: complete remission; CT: chemotherapy; BMT: bone marrow transplantation.

Table 3 – Distribution of events according to MRD at D15 by MFC.

Events	<0.1%		0.1–10%		>10%		Total	
	No.	%	No.	%	No.	%	No.	%
Total	23	30.7	39	52	13	17.3	75	100
Morphology								
M1	18		26		2		46	61.3
M2	5		12		0		17	22.7
M3	0		1		11		12	16
Relapse	2		10		4		16	21.3
BM		7		2				
Testis	1		1					
CNS		2						
BM + CNS	1			1				
BM + lymph nodes			1					
Deaths	2		8		7		17	22.6
Disease		4		5				
In CR after CT	2		4		1			
In CR after BMT			1					

MRD: minimal residual disease; MFC: multiparametric flow cytometry; BM: bone marrow; M1: BM blasts < 5%; M2: BM blasts 5% to < 25%; M3: BM blasts ≥ 25%; CNS: central nervous system; CR: complete remission; CT: chemotherapy; BMT: bone marrow transplantation.

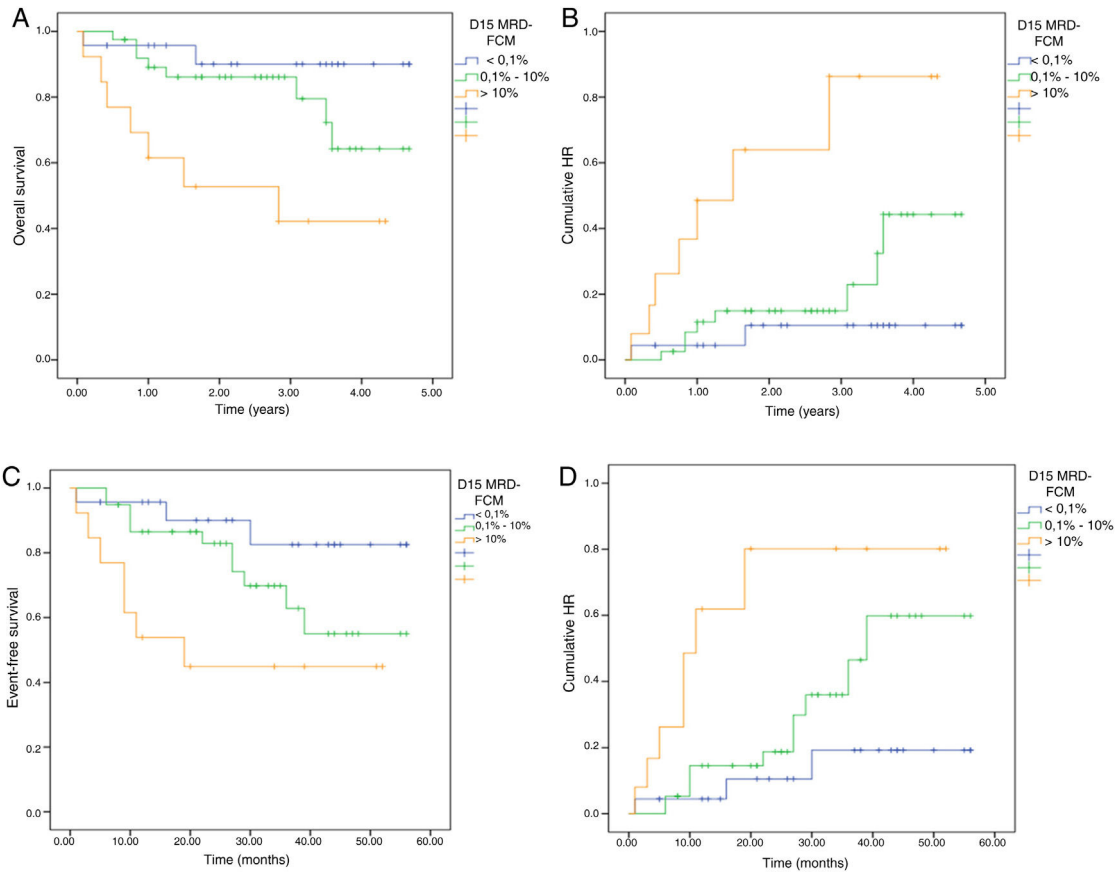

Figure 2 – (A) Estimated overall survival according to MRD at day 15 (D15) of induction by flow cytometry and (B) cumulative hazard ratio (HR) for death according to MRD at D15. (C) Estimated event-free survival according to MRD at day 15 (D15) of induction by flow cytometry and (D) cumulative HR for relapse according to MRD at D15.

All patients underwent morphological evaluation at D15 and at the end of the induction. At D15, 46 (61.3%) patients had an M1 marrow (<5% blasts), 17 (22.7%) had an M2 marrow (5% to <25% blasts) and 12 (16.0%) had an M3 marrow (≥25% blasts).

The estimated 4-year overall survival of patients with D15 MRD-MFC <0.1%, 0.1–10%, and >10% was 90.0%, 64.2%, and 42.2%, respectively (Figure 2A), while the cumulative HR for death at 4 years was 10.5%, 44.2%, and 86.2%, respectively (Figure 2B) ($p = 0.003$; log-rank test).

The estimated 4-year event-free survival of patients with D15 MRD-MFC <0.1%, 0.1–10%, and >10% was 82.5%, 55.0% and 44.9%, respectively (Figure 2C), while the cumulative HR for relapse was 19.2%, 59.8% and 80.1%, respectively (Figure 2D) ($p = 0.014$; log-rank test).

Other variables, such as the NCI criteria, morphology at D15 and response to corticosteroid therapy, also showed prognostic significance. None of the patients with the MRD < 0.1% at D15 showed poor response to corticosteroids. The event-free survival rates according to these variables are shown in Table 4. However, the use of multivariate Cox regression models was not possible due to the small number of cases and outcomes.

Discussion

Treatment of ALL is one of the most complex therapies in anticancer programs. Children with ALL are classified and treated according to relapse risk groups.[19] Risk groups are characterized based on the combination of disease features and prognostic factors. This allows therapists to intensify the therapeutic approach for patients at higher risk of relapse, while providing less toxic therapy for those with a high probability of cure.[20]

Clearance of leukemic cells from peripheral blood at D8 of treatment and from bone marrow at D15 of treatment and at the end of the induction is an important factor in assessing the risk of disease relapse.[21] A poor response to corticosteroids has been observed in less than 10% of patients, and this parameter has been used for risk stratification.[5] In our sample, 13.3% of patients showed a poor response to corticosteroids (Table 4).

In addition to the analysis of peripheral blood after 1 week of the induction therapy, the analysis of bone marrow smears during and after the induction therapy was also used to assess early treatment response.[22] However, the morphological

Table 4 – Distribution of variables according to morphology at D15, D8, and NCI criteria.

Variables	n	%	EFS (%)	p
Morphology at D15				(0.001; log-rank test)
M1	46	61.3	74.3	
M2	17	22.7	67.7	
M3	12	16	31.1	
D8				(<0.001; log-rank test)
<1,000 blasts/mm^3	65	86.7	78	
>1,000 blasts/mm^3	10	13.3	20	
NCI				(<0.001; log-rank test)
LR	44	58.7	94.8	
HR	31	41.3	28.3	

EFS: event-free survival; NCI: National Cancer Institute; D8: day 8 of induction; D15: day 15 of induction; M1: bone marrow blasts < 5%; M2: bone marrow blasts 5% to < 25%; M3: bone marrow blasts ≥ 25%; LR: low-risk; HR: high-risk.

evaluation of bone marrow by light microscopy is a low-sensitivity technique. For this reason, the use of more sensitive analytical methods is currently recommended, such as the MRD.[23]

The MRD can be detected by several techniques, including the MFC of aberrant immunophenotypes, analysis of rearranged immunoglobulin (Ig) and T-cell receptor (TCR) genes by the PCR and analysis of fusion gene transcripts (RNA), also by the PCR.[8]

The main advantages of the MRD measurement by the MFC include rapid cell processing, low cost, wide availability and accurate quantification of abnormal cells. The main disadvantages are the need for high technical expertise, variable sensitivity due to similarities between normal regenerative cells and malignant cells and the possibility of phenotypic changes after the initiation of treatment due to clonal evolution of leukemic cells or to the drugs used.[8] An additional advantage is that the MFC can detect intact cells, in contrast to the PCR. Both techniques have limitations because, similar to the dominant immunophenotype, Ig/TCR gene rearrangements may also evolve over time and thus become unrecognizable.[22] However, the MFC is less sensitive than the PCR. There have been discussions on how to lower the detection limit to $<10^{-5}$–10^{-6}, attempting to achieve a sensitivity similar to that of the PCR techniques.[8]

Future trends indicate that treatment will be increasingly tailored to the individual. Despite significant historical advances, much is yet to be improved in this field. The PCR technology is not available in most cancer treatment centers in Brazil. Therefore, because the MFC is less expensive and more accessible, studies have been conducted with the purpose of improving the MRD analysis technique by the MFC in ALL, especially in developing countries such as Brazil.

Childhood ALL treatment protocols recommend the MRD monitoring at multiple time points. Several studies have shown that detection of the MRD at the end of the induction is an important predictor of outcomes in children and adolescents with B-ALL.[24] However, an earlier assessment of the MRD has also been investigated and its prognostic value has been confirmed. Measurement of bone marrow MRD by the MFC approximately 2 weeks after the initiation of the induction chemotherapy (D15) has also provided additional benefit to the risk stratification.[10]

In our study, the importance of the MRD at D15 to intermediate-risk group stratification was confirmed by the comparison of protocols that include the MRD vs. protocols that do not include the MRD (Table 1). Of the 23 patients and of the 21 patients classified as 'low-risk' by the BFM 95 and IC-BFM 2002 protocols, respectively, only 8 were classified as 'low-risk' by the IC-BFM 2009 protocol. The remaining patients were classified as 'intermediate-risk' by the IC-BFM 2009 protocol. Despite limited sample size, there is little doubt that this is due to the inconclusiveness of the MRD measurement at D15 in the IC-BFM 2009 classification system, since the results of morphological evaluation at D15 showed only M1 and M2 marrow cases, with no cases of M3 marrow (Table 3). If, at this time, only morphological evaluation had been considered, the agreement between low-risk groups would have been higher, which did not occur.

Analysis of our data suggests D15 MRD-MFC < 0.1% as a cut-off point for patients with more favorable outcomes and D15 MRD-MFC > 10% for patients with unfavorable outcomes. These findings support data from the literature demonstrating the importance of measuring MRD at D15 by the MFC as a strong early predictor of prognosis.[10] However, we cannot state in the present study that the MRD measured at D15 by the MFC was the major predictor of prognosis due to the small number of cases and outcomes in the multivariate analysis. This, however, was demonstrated by the AIEOP-BFM ALL 2000 study.[10]

In the present study, patients with D15 MRD-MFC < 0.1% also had longer overall and event-free survival and lower cumulative risk of death or relapse (Figure 2). Although survival was estimated at 4 years due to the study length, the results did not differ significantly from those reported in the AIEOP-BFM ALL 2000 study, in which survival was estimated at 5 years. For our patients with D15 MRD-MFC < 0.1%, the estimated event-free survival rate was 82.5% at 4 years, against 89.9% at 5 years for this patient group in the AIEOP-BFM ALL 2000 study.[10] This demonstrates that the MRD measured at D15 by the MFC may contribute to further refining prognostic classification and suggests that this group of patients may benefit from dose reduction during induction therapy.[25]

Furthermore, our patients with the MRD-MFC < 0.1% at the end of the induction were less likely to die, while the MRD-MFC 0.1–10% was significantly associated with relapse and the MRD-MFC > 10% was associated with death and relapse at the

end of induction. These findings are consistent with the literature in demonstrating that the absence of the MRD at the end of the induction is the major predictor of a favorable outcome and that detectable MRD levels at the end of the induction indicate increased risk of relapse and poorer survival.[7]

In a study conducted at St. Jude Children's Research Hospital, the MRD was assessed by the MFC at D19 of the induction and the prognostic value of the MRD on D19 bone marrow defined by the MFC was superior to that defined by morphological studies.[26] In a study conducted by the same research group, the MRD measurement at D19 was particularly useful for patients with favorable presenting features. It should be noted that the MRD was measured by both the MFC and PCR and the results were generally concordant.[23]

The Children's Oncology Group reported a very favorable prognosis for patients with B-ALL, low-risk criteria and favorable cytogenetic abnormalities who had MRD levels measured by the MFC below 0.01% at D8 in peripheral blood and at the end of the induction in bone marrow. Presence of the MRD at these time points was associated with shorter event-free survival in all risk groups.[7]

A study investigating the prognostic impact of the MRD in peripheral blood at D15 of the induction identified a large group of patients with an excellent prognosis and added prognostic information to risk stratification based on the MRD at the end of the induction and at week 12 of the treatment. However, the MRD was assessed at D15 by the PCR. The MRD assessment by the PCR is time-consuming and requires at least 2 weeks (but usually 4), which would hinder the implementation of an early treatment intervention based on D15 MRD-PCR.[27]

Defining the most favorable prognostic group as early as possible, such as at D15 of the induction after 1 week of corticosteroids and 1 week of chemotherapy plus corticosteroids, is extremely useful and beneficial to patients, as it offers the possibility of early chemotherapy de-escalation, for example, by decreasing the dose of anthracyclines used in the induction therapy in the BFM-based protocols.[27] This may contribute to reducing the late effects associated with long-term treatment, such as leukemia treatment.

Conclusion

The present study is one of the few Brazilian studies to demonstrate the influence of the MRD inclusion on the risk classification of pediatric patients with B-ALL. Another study demonstrated the role of peripheral blood MRD at day 8 of the induction therapy.[28] The MRD measurement by the MFC is a feasible approach in our routine practice to refine the risk stratification of our patients. Our data suggest that the MRD at D15 has an influence on risk classification, being particularly useful for stratification of patients with a more favorable prognosis, as demonstrated in the literature.[25]

In conclusion, we recognize that this study has limitations that are inherent in its retrospective design and is based on data from a single Brazilian referral institution for cancer treatment, with a limited sample size and study period. Nevertheless, our findings are similar to those reported in the literature on the impact of the MRD analysis. Further preferably prospective and cooperative studies are needed to better characterize our population, improve our knowledge and ensure an increasingly tailored treatment, as recommended by international studies.

Conflicts of interest

The authors declare no conflicts of interest.

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