Assessment of Minimal Residual Disease in Childhood Acute Lymphoblastic Leukemia: A Multicenter Study From Turkey

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Summary: Assestment of minimal residual disease (MRD) in childhood acute lymphoblastic leukemia (ALL) is of utmost importance both for risk classification and tailoring of the therapy. The data of pediatric ALL patients that received treatment with Berlin-Frankfurt-Münster (BFM) protocols were retrospectively collected from 5 university hospitals in Turkey. Of the 1388 patients enrolled in the study 390 were treated according to MRD-based protocols. MRD assestment was with real time quantitative polymerase chain reaction (qPCR) in 283 patients and with multiparametric flow cytometry (MFC)-MRD in 107 patients. MRD monitoring had upstaged a total of 8 patients (2%) from intermediate risk group to high-risk group. Univariate analysis revealed age 10 years or above, prednisone poor response, PCR-MRD $\geq 10^{-3}$ on day 33 and on day 78 as poor prognostic factors affecting event-free survival (EFS). Detection of $>10\%$ blasts on day 15 with MFC (MFC-high-risk group) was not shown to affect EFS and/or overall survival (log-rank $P=0.339$). Multiple logistic regression analysis revealed PCR-MRD $\geq 10^{-3}$ on day 78 as the only poor prognostic factor affecting EFS (odds ratio: 8.03; 95% confidence interval: 2.5-25; $P=0.000$). It is very important to establish the infrastructure and ensure necessary standardization for both MRD methods for optimal management of children with ALL.

Key Words: acute lymphoblastic leukemia, childhood, minimal residual disease

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Cure rates for childhood acute lymphoblastic leukemia (ALL) have increased dramatically for the last 40 years. Successful implementation of multiagent chemotherapy regimens with better management of complications and advanced supportive care, led to higher survival rates, even in developing countries. However, relapse still remains the major cause of treatment failure in ALL.[1–10] Various elements including host factors, immunophenotype, certain genetic alterations and treatment response have been shown to have roles in predicting relapse and therefore they are used for risk stratification.[1,11,12] In this regard detection of minimal residual disease (MRD) has become the most powerful factor in measuring in vivo treatment response as blasts that are not detectable by conventional pathologic methods can be assessed by more sensitive MRD detection techniques. Accordingly, modern ALL protocols use MRD-based risk classification for better evaluation of treatment response and tailoring of the therapy.[4,6,7,11–15]

Several methods have been developed for MRD detection; the most commonly used MRD techniques are multiparametric flow cytometry (MFC) and immunoglobulin/T-cell receptor (Ig/TCR) quantitative polymerase chain reaction (qPCR). There are also newly emerging and even more sensitive other techniques like droplet digital PCR and high-throughput next generation sequencing, but their clinical use is limited for the time being.[15–18]

MFC is a rapid, sensitive and relatively inexpensive method; but the challenge of standardization across laboratories is a major disadvantage of this method. Nevertheless, there have been attempts for standardization of this method.[19,20]

Quantitative PCR for Ig/TCR rearrangements is a highly sensitive method and there are well-defined standard guidelines for application and interpretation, but this method is time consuming, labor intensive and expensive.[16]

Childhood ALL is treated in many centers in our country and most of them use Berlin-Frankfurt-Münster (BFM) based protocols for treatment. These protocols use either MFC or qPCR or both for MRD analysis, but a standard MRD follow-up is not uniformly applied throughout centers.

In this study, we wanted to evaluate the changes in the risk group classification and define the treatment response in Turkish children with ALL in whom MRD studied either with real time qPCR or MFC. We also aimed to compare the treatment results with earlier patients in whom MRD could not be studied.

METHODS

Patients

Between April 1993 and March 2019, 1388 patients with newly diagnosed ALL from 5 university hospitals in Turkey treated according to BFM protocols (ALL BFM-90, ALL BFM-95, ALL BFM 2000, and ALLIC-BFM 2009) were enrolled. Of the 1388 patients 390 were treated according to MRD-based protocols (ALL BFM 2000 and ALLIC-BFM

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2009) between May 2009 and March 2019. MRD assessment was with real time qPCR in 283 patients that were treated according to ALL-BFM 2000 protocol and with MFC in 107 patients that were treated according to ALLIC-BFM 2009 protocol.

MRD Analysis

Real Time qPCR-MRD

All PCR-MRD samples were analyzed in Germany (Heidelberg or Frankfurt) as PCR-MRD analysis cannot be performed in Turkey. MRD-PCR was only sent from patients whose families could afford the price. Bone marrow samples for MRD were obtained at initial diagnosis and at the three consecutive follow-up time points day 15, day 33 (TP1) and day 78 (TP2) and sent to genetic laboratories in Heidelberg or Frankfurt (Germany) according to preference of centers. MRD levels were routinely determined by RQ-PCR analysis of Ig and/or TCR gene rearrangements. PCR-MRD reaction targets were tested for specificity and sensitivity with the aim of selecting at least 2 targets with a sensitivity of $\leq 10^{-4}$ and a quantitative range of at least 10^{-4} for one target and at least 5×10^{-4} for the second target. If MRD levels differed between the 2 markers, the highest MRD level was chosen for the final MRD assessment. Cell sample isolation, and the identification of markers for MRD evaluation have been reported in detail elsewhere.[4,7,21–23]

MFC-MRD

Three centers which were approved by European ALLIC-BFM center were able to study MFC-MRD in their own immunology laboratory. The expenses are covered by the social health insurance in Turkey. The bone marrow samples obtained from the cases on the 15th day of treatment, were immediately processed with specific fluorochrome (fluorescein isothiocyanate [FITC], phycoerythrin [PE], peridinin chlorophyll protein [PerCP], phycoerythrin-cyanin 5 [PE-Cy5], PE-Cy7, allophycocyanin [APC], APC-Cy7, Alexa flour 700 [A×F700], Blue violet 510 [BV510], BV650)conjugated monoclonal antibodies: CD4 (SK3-FITC), CD7 (MT701-APC), CD10 (HI10a-PE-Cy7), CD19 (SJ25C1-APC), CD20 (L27-FITC, -APC-Cy7), CD34, (8G12-FITC, -PE), CD45 (2D1-PerCP, -Amcyan), TdT (E171519-FITC) from BD Biosciences (San Jose, USA), CD3 (UCHT1-A×F700), CD8 (SK1-APC-Cy7), CD38 (HIT2-A×F700), CD58 (1C3/AICD58.6-FITC) from BD Pharmingen (USA), CD11a (HI111-PE, -BV650) from BD Pharmingen or BD Horizon (USA), CD99 (3B2/TA8-PE) from EXBIO Praha, a.s CZ or eBioscience\Thermo Fisher Scientific (USA) and CD5 (UCHT2-PE-Cy5 or L17F12-PE-Cy7) from BD Pharmingen or BioLegend (USA) according to the phenotypic characteristics of disease and ALL IC-Berlin-Frankfurt-Münster (BFM) 2009 Standard Operating Procedure.[6] Briefly bone marrow cells were directly stained by monoclonal antibodies using the "stain, lyse and then wash" approach. After incubation at room temperature for 15 minutes in the dark, nucleated cells were prepared using 2 mL of a commercially available red cell lysing solution (BD Bioscience). Cells were washed 2 times with 2 mL of PBS, resuspended in 500 mL of PBS. For detection of cytoplasmic markers (CD3 and TdT) cells were processed with cell permeabilization kit (Fix-Perm An Der Grub Bio Research GMBH, Austria). To exclude residual nonnucleated erythroid cells, thrombocytes, or debris added SYTO 16 (emission at 518 nm; Molecular Probes, Leiden, The Netherlands) the cell-permeant, nucleic acid fluorochrome and subsequently data acquired and analyzed using FACSCalibur or FACS Aria II (BD Biosciences, USA) or Novocyte (ACEA Biosciences Inc., USA) flow cytometers with Cell-Quest Pro or FACSDiVa (BD Biosciences) or Novoexpress (ACEA Biosciences Inc.) software, respectively. Minimum 300,000 nucleated cells were acquired, gates were set in CD19 or CD7 positive cells. The residual blast cells were determined as percentage and were classified as low risk with <0.1%, intermediate risk (IR) between 0.1% and 10%, and high-risk (HR) with > 10%.

Risk Stratification

The diagnosis of ALL was based on the morphology, immunophenotyping, cytogenetics, and molecular features of leukemia cells. Risk group stratification according to ALL BFM-90, ALL BFM-95, ALL BFM-2000, and ALLIC-BFM 2009 protocols are as follows.[5–7,24–26]

ALL-BFM-90

Standard risk group (SR): BFM-RF <0.8 and no CNS involvement and no T-ALL, and no HR group criteria.

Medium risk group (MR): BFM-RF <0.8 and/or CNS involvement and/or T-ALL, and no HRG criteria.

HR: prednisone poor response (PPR) and/or no CR after induction phase Protocol IA (day 33) and/or evidence of t(9;22) (or *BCR/ABL*).

ALL-BFM-95

SR: initial white blood cells (WBC) <20,000/μL and age at diagnosis 1 year or above and below 6 years and no T-ALL and no HR criteria.

MR: initial WBC > 20,000/μL and/or age at diagnosis below 1 or above 6 years, and/or T-ALL and no HR criteria.

HR: PPR and/or no CR after induction phase Protocol IA (day 33) and/or evidence of t(9;22) (or *BCR/ABL*) and/or evidence of t(4;11) (or *MLL/AF4*).

ALL-BFM 2000

PCR-MRD is used for risk stratification.

MRD SR: MRD negativity on treatment day 33 (TP1) and 78 (TP2) and no other HR criteria.

MRD IR: positive MRD at either one of both time points but at a level $<10^{-3}$ at TP2 and no other HR criteria.

MRD HR: positive MRD at TP2 ($\geq 10^{-3}$) and/or PPR and/or non-remission to induction treatment and/or evidence of t (9;22) (or *BCR/ABL*) and/or evidence of t(4;11) (or *MLL/AF4*).

ALLIC-BFM 2009

SR: initial WBC <20,000/μL and age at diagnosis 1 year or above and below 6 years and no T-ALL and if available FC MRD < 0.1% or M1/M2 marrow on day 15 and no M2/M3 marrow on day 33 and no HR criteria.

HR: IR and if available FC MRD <0.1% or M3 marrow on day 15, SR if available FC MRD > 10% and/or PPR, and/or M2/M3 marrow on day 33 and/or evidence of t(9;22) (or *BCR/ABL*) and/or evidence of t(4;11) (or *MLL/AF4*) and/or hipodiploidy (at least one criterion must be fullfilled).

IR: all patients who are not stratified to SR or HR.

Treatment Protocol

The ALL BFM-90 treatment protocol was administered between 1993 and 1996 and thereafter the ALL BFM-95 in all centers until 2009. ALL-BFM 2000 or ALLIC-BFM 2009 protocols were administered after the year 2009 depending on

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the preferred MRD technique of the center. Briefly, all protocols consist of induction, consolidation, reinduction and maintenance phases and CNS-directed therapy. The details of the treatment protocols are described in detail elsewhere.[5–7,24–26]

Statistical Analysis

Event-free survival (EFS) was defined as the time from diagnosis to the date of the last follow-up in complete remission or the first event. "Events" were nonresponse to therapy, abandonment of treatment, relapse, second malignant neoplasm or death from any cause, whichever occurred first. Overall survival (OS) was defined as the time from initiation of treatment to death from any cause. EFS and survival curves were estimated according to Kaplan-Meier and compared according to log-rank test. Univariate and multivariate Cox regression analyses were also performed to determine the prognostic implications of certain variables. Comparison of continuous and categorical variables was performed by Mann-Whitney U test and the χ^2 test. A P-value of <0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS 24.

RESULTS

MRD was studied in a total of 390 patients since May 2009. The MRD method was real time qPCR in 283 patients and MFC in 107 patients. The characteristics of the patients are given in Table 1. Parameters including age, sex, initial leukocyte count and day 8 prednisone response did not differ between groups. The distributions of risk groups differed among MRD and non-MRD groups. More patients were classifed as HR in the non-MRD group with respect to MRD groups. T-cell ALL immunophenotype was significantly higher in non-MRD group. In contrast, less patients were found to have *ETV6/RUNX* fusion gene in non-MRD group compared with MRD groups.

MRD monitoring had upstaged 7 patients (2.4%) from IR to HR in the PCR group. In the MFC-MRD group one patient was upstaged from IR to HR and 5 patients were upstaged from SR to IR. In summary, a total of 8 patients (2%) were upstaged to HR in the whole MRD group. None of the patients' risk group was downstaged according to MRD results.

The 5-year EFS was 82% in MRD group and 78% in the non-MRD group ($P=0.251$) and 5-year OS was 87.4% in MRD group and 85.1% in the non-MRD group ($P=0.152$). Both OS and EFS did not differ significantly between MRD and non-MRD groups.

However, analysis of survival rates according to MRD method revealed differences (Fig. 1). The 5-year EFS was found to be significantly higher in the PCR-MRD group.

The outcomes of SR, IR and HR groups in PCR-MRD and MFC-MRD groups are shown in Figure 2. In the PCR-MRD group; 5-year EFS was 92%, 82%, and 62% in the SR, IR, and HR groups, respectively ($P=0.000$). The 5-year OS was 95%, 90%, and 60% in the SR, IR, and HR groups, respectively. The 5-year OS was similar in SR and IR groups but significantly lower in the HR group ($P=0.000$). In the MFC-MRD group; 5-year EFS was 85%, 68%, and 68% in the SR, IR, and HR groups, respectively ($P=0.533$). It was important to note that the EFS rates did not differ significantly between risk groups in the MFC-MRD group. The 5-year OS was 89%, 85%, and 79% in the SR, IR, and HR groups, respectively. Although the OS was low in the HR group, it was not statistically significant ($P=0.521$).

TABLE 1. Characteristics of the Patients

	PCR-MRD	MFC-MRD	Non-MRD Group	P
Number of patients	283	107	998	
Follow-up months, median (range)	46 (8-104)	45 (8-120)	111 (8-297)	
Age (mo), median (range)	58.8 (6-211)	60 (11-210)	66 (8-216)	0.149
Sex, n (%)				
Male	171 (60.4)	58 (54.2)	602 (60.3)	0.461
Female	112 (39.6)	49 (36.8)	396 (39.7)	
Initial leukocyte count (10^9/L), n (%)				
< 50	215 (76)	83 (79)	785 (79.5)	
50-100	28 (9.8)	11 (10.5)	97 (9.8)	0.585
> 100	40 (14.2)	11 (10.5)	105 (10.7)	
Immunophenotype, n (%)				
Pre-B-cell	250 (88.3)	97 (91.5)	805 (83.1)	**0.013**
T-cell	33 (11.7)	9 (8.5)	164 (16.9)	
Risk group, n (%)				
Standard	107 (37.8)	23 (21.5)	170 (17)	**0.000**
Intermediate	142 (50.2)	67 (62.6)	606 (60.7)	
High	34 (12)	17 (15.9)	222 (22.3)	
Treatment protocol, n (%)				
ALL-BFM-90				
ALL-BFM-95			360 (36)	
ALL-BFM 2000	283 (100)		549 (55)	
ALLIC-BFM 2009		107 (100)	89 (9)	
Day 8 prednisone response, n (%)	n = 283	n = 101	n = 955	
Good	242 (85.5)	87 (86.1)	854 (89.4)	0.147
Poor	41 (14.5)	14 (13.9)	101 (10.6)	
Fusion genes, n (%)	n = 221	n = 29	n = 541	
BCR/ABL1	10 (4.5)	2 (6.8)	28 (5.1)	0.846
MLL/AF4	7 (3.16)	1 (3.4)	14 (2.5)	0.891
ETV6/RUNX	50 (22.6)	7 (24.1)	54 (10)	**0.000**

Statistical significance values are in bold.
MFC indicates multiparametric flow cytometry; MRD, minimal residual disease; PCR, polymerase chain reaction.

The incidence of relapse was investigated in patients two years after completion of ALL therapy. Thirteen of 139 (9.3%) patients relapsed in PCR-MRD group and 5 of 43 (11.6%) patients relapsed in MFC-MRD group; the difference between 2 groups was not statistically significant ($P=0.662$). Similarly, the relapse rates between MRD and non-MRD group did not differ significantly. Eighteen of 182 (9.8%) patients relapsed in MRD group while 64 of 722 (8.9%) patients relapsed in non-MRD group ($P=0.667$).

Univariate analysis revealed age 10 years or above, PPR, PCR-MRD $\geq 10^{-3}$ on day 33 and on day 78 as poor prognostic factors affecting EFS and initial leukocyte count $\geq 50\times10^9$/L, PPR, PCR-MRD $\geq 10^{-3}$ on day 33 and on day 78 as poor prognostic factors affecting OS (Table 2). However, detection of > 10% blasts on day 15 with MFC (MFC-HR group) was not shown to affect EFS and/or OS (log-rank $P=0.339$). Multiple logistic regression analysis revealed PCR-MRD $\geq 10^{-3}$ on day 78 as the only poor prognostic factor affecting EFS (odds ratio: 8.03; 95% confidence interval: 2.5-25; $P=0.000$) and PPR (odds ratio: 4.29; 95% confidence interval: 1-18.2; $P=0.049$) and PCR-MRD $\geq 10^{-3}$ on day 78 (odds ratio: 5.2; 95% confidence interval: 1.1-23.5; $P=0.03$) as poor prognostic factors affecting OS (Table 3).

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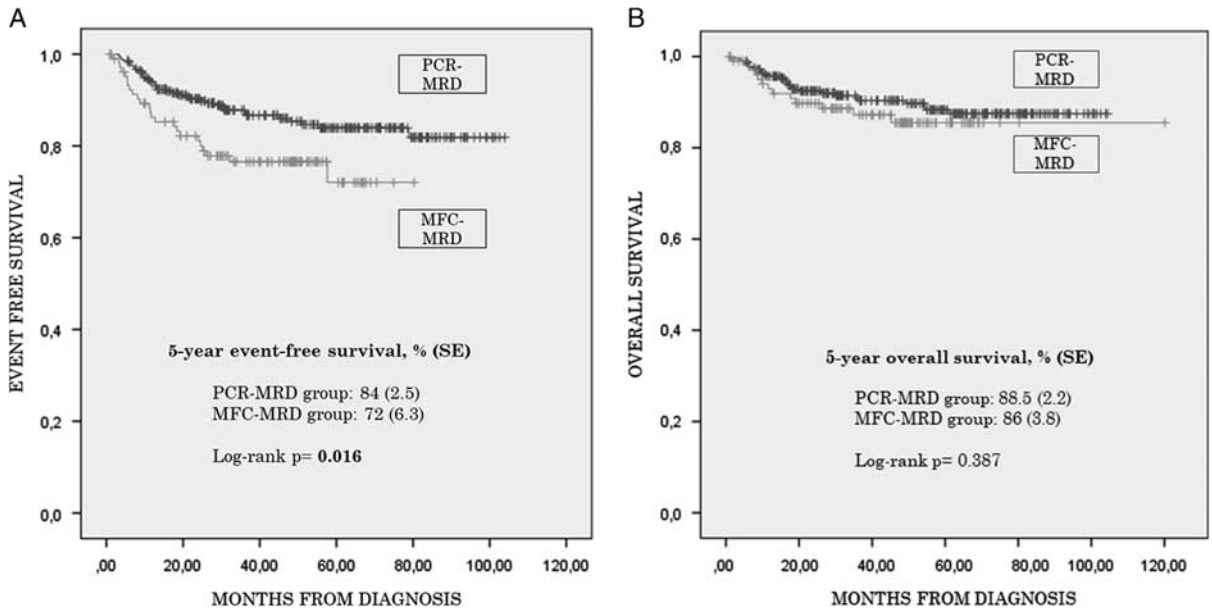

FIGURE 1. Event-free survival (A) and overall survival (B) in polymerase chain reaction (PCR)-minimal residual disease (MRD) and multiparametric flowcytometry (MFC)-MRD groups.

DISCUSSION

Numerous studies have shown that MRD is the single most important prognostic factor in childhood ALL. Modern ALL protocols use MRD-based risk stratification both for intensification and de-escalation of treatment.[4,6,7,11–13,25,27,28] However, problems in infrastructure and standardization of MRD are major obstacles to the widespread application of this method all over the world.

ALL has been treated successfully in many centers of Turkey, but unfortunately MRD has not been fully incorporated into treatment protocols of all centers because of technical and economical problems.[8,9] Some centers developed their own immunology laboratory for MFC-MRD measurement. In this regard, ALLIC-BFM 2009 protocol has been widely used by many centers as it only uses 15th day bone marrow for MFC-MRD analysis. In contrast, some centers prefer to use ALL BFM 2000 protocol and send bone marrow samples abroad for PCR-MRD analysis. In this study we investigated the characteristics and outcome of children with ALL in this MRD era and also compared the results with those whom MRD was not studied.

In the AIEOP BFM ALL 2000 study, among 3184 patients, 42% were PCR-MRD SR, 52% were PCR-MRD IR and 6% were PCR-MRD HR.[7] The percentage of HR patients was 2 times higher in our PCR-MRD group (12%) compared with AIEOP BFM 2000 study. Basso et al[6] defined the risk of relapse based on MFC-MRD on day 15 bone marrow among 815 patients and classified 42% as SR, 46% as IR and 11% as HR. The distribution of risk groups was also different between MRD and non-MRD groups in our study. Of the 998 patients enrolled in the non-MRD group 17%, 61%, and 22% of patients were assigned to SR, IR, and HR groups, respectively. One explanation for this result is that; in the past, cytogenetic analysis and molecular cytogenetic analysis could not be performed for all patients so physicians had a tendency to classify more patients as HR not to undertreat. However, this 'safe approach' is not innocent as it is already known that treatment-related toxicity is higher in the HR groups compared with other groups.[29–31] In this context, along with classic risk factors, better stratification of risk groups with more sensitive methods like MRD carries great importance to decrease treatment-related toxicity.

Personalized treatment based on MRD allows physicians to identify patients who require intensified treatment as well as those who can be successfully treated with low-intensity regimens.[28,32–39] Current treatment regimens follow MRD results for intensification of treatment in the presence of some factors other than classic poor prognostic factors. In our study, MRD monitoring had upstaged a total of 8 patients (2%) from IR to HR, of which 7 were in the PCR group, and 1 patient was in the MFC-MRD group. Those patients did not have classic poor prognostic factors but we do not know whether they had other factors like Philadelphia chromosome-like ALL, intrachromosomal amplification of chromosome 21 or *IKZF1*^{plus} deletion. Several leukemia study groups have attempted to reduce treatment intensity with a randomized approach in patients with most favorable prognosis based on MRD levels. The results are variable because of different treatment approaches and biological heterogeneity, yet data suggest that treatment reduction is feasible in specific subgroups.[28,37–39] Of note, none of our patients' risk group were downstaged based on MRD results.

The 5-year EFS and OS rates were 82% and 87.4% in MRD group and 78% and 85.1% in the non-MRD group, respectively. Both OS and EFS did not differ significantly between MRD and non-MRD groups. Similarly, the incidence of relapse did not differ between MRD and non-MRD groups. Our results are comparable with AIEOP BFM ALL 2000 study where 7-year rates for EFS and OS were 80.7% and 92.8%, respectively in a total of 3184 patients that were stratified by PCR-MRD.[7] In our previous study including ALL patients before 2009 (non-MRD era), 5-year EFS and OS were found to be 78.4% and 79.9%, respectively.[9] However, analysis of survival rates according to MRD method revealed differences in the current study. The OS rates did not differ between PCR-MRD and MFC-MRD groups but 5-year EFS was found to be

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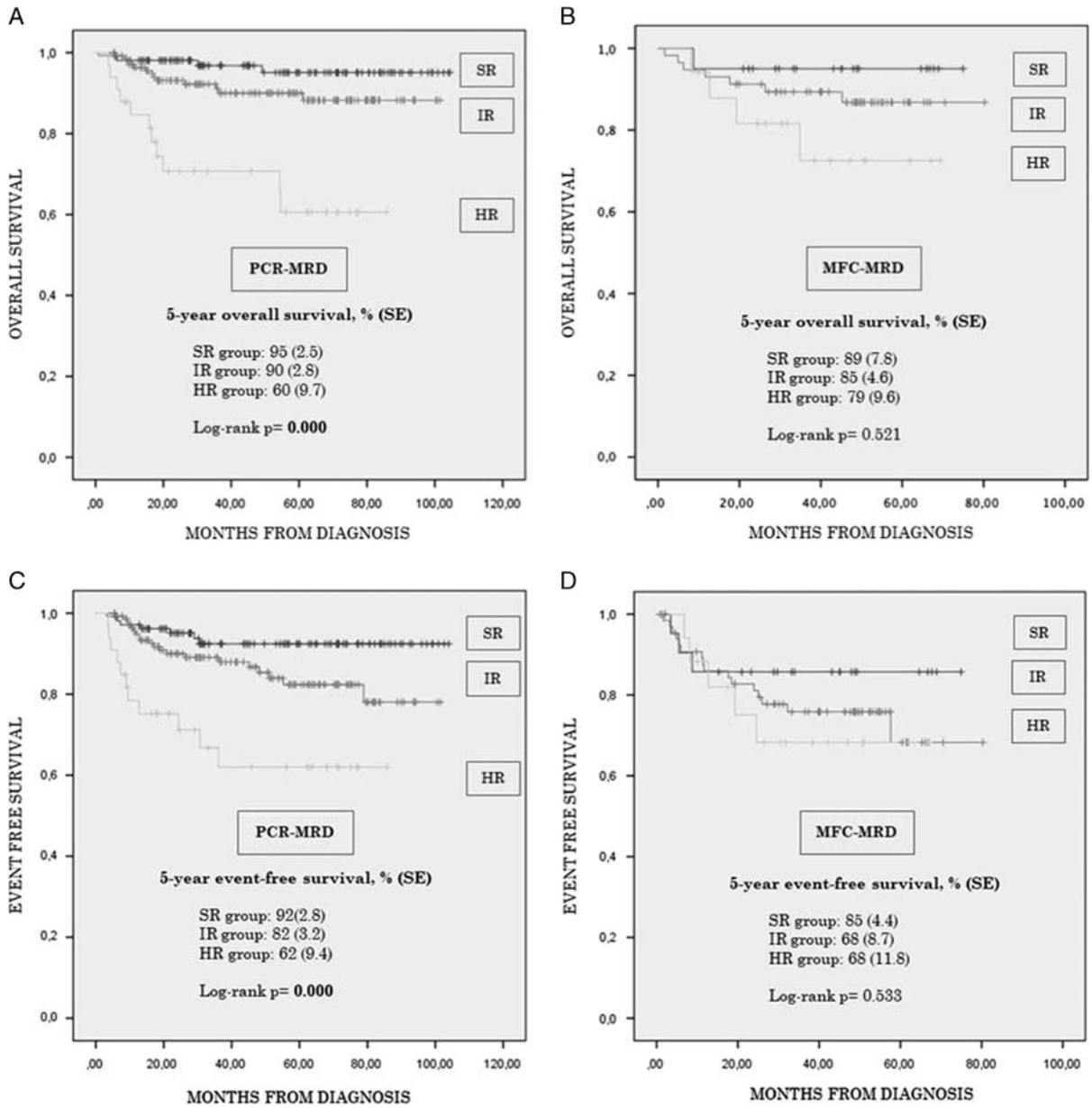

FIGURE 2. The outcomes of standard, intermediate and high-risk group in polymerase chain reaction (PCR)-minimal residual disease (MRD) (A, C) and multiparametric flow cytometry (MFC)-MRD groups (B, D).

significantly higher in the PCR-MRD group. Further analysis according to risk group revealed significant differences between risk groups in PCR-MRD group. The outcome was excellent in SR group of PCR-MRD patients with 5-year EFS and OS rates of 92% and 95%, respectively. This result was no surprise as early MRD negativity was defined as the strongest predictor for excellent outcome in the previous studies.[7,11,38] The 5-year EFS was 92.3% in SR group in the AIEOP BFM ALL 2000 study, similar to our study.[7] In contrast, high levels of MRD on day 78 (MRD-PCR HR group) has been defined as predictive for poor outcome.[7,25] The 5-year EFS was 50.1% in HR group in the AIEOP BFM ALL 2000 study.[7] In our study, 5-year EFS rate was 62% in HR group of PCR-MRD; the rate was significantly lower compared with other risk groups. Our findings in the PCR-MRD group were in accordance with the current literature however the risk group stratification did not appear discriminative among MFC-MRD group in our study. Univariate analysis revealed initial age 10 years or above, PPR, MRD-PCR $\geq 10^{-3}$ on day 33 and on day 78 (MRD HR group) as poor prognostic factors affecting EFS, however detection of $>10\%$ blast on day 15 with MFC (MFC-HR group) did not appear to affect EFS or OS. Basso et al studied MFC-MRD on day 15 bone marrow of 831 ALL patients and described 5-year EFS as 46.1% in those who had at least 10% blasts (HR group) in bone marrow.[6] The Children's Oncology Group showed that positive ($\geq 0.01\%$) MFC-MRD on day 29 (end of induction) was associated with poorer outcome and day 29 MRD was the independent prognostic variable in the multivariate analysis.[40] In contrast it is known that MFC-MRD method has some limitations in terms of standardization. Difficulties in obtaining

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TABLE 2. Factors Affecting Even-free Survival and Overall Survival in MRD Group

Variables	5 y-EFS, % (SE)	*P*	5 y-OS, % (SE)	*P*
Sex				
Male	78.8 (3)	0.062	86.7 (2.6)	0.546
Female	84.2 (3.6)		88.6 (2.8)	
Age (y)				
<10	83.3 (2.5)	**0.039**	89 (2.1)	0.06
≥ 10	73.8 (5.3)		81.9 (4.5)	
Initial leukocyte (10^9/L)				
<50	83.8 (2.4)	0.096	89.8 (1.9)	**0.046**
≥ 50	72.9 (5.4)		77.5 (5.5)	
Immunophenotype				
Pre-B-cell	81.1 (2.5)	0.46	89.6 (2)	0.055
T-cell	77.7 (6.6)		79.3 (7)	
Day 8 prednisone response				
Good	82.7 (2.4)	**0.013**	90.2 (1.9)	**0.000**
Poor	69.9 (6.7)		74.9 (6.6)	
Day 15 MFC-MRD				
$<10\%$	80.6 (4.5)	0.422	86.2 (4)	0.339
$\geq 10\%$	76.2 (9.4)		79.1 (9.6)	
Day 33 PCR-MRD				
$<10^{-3}$	91.9 (2.8)	**0.009**	94.5 (2.5)	**0.011**
$\geq 10^{-3}$	78.4 (3.7)		84 (3.3)	
Day 78 PCR-MRD				
$<10^{-3}$	86.8 (2.4)	**0.000**	91.9 (1.9)	**0.000**
$\geq 10^{-3}$	40.8 (13.6)		38.1 (14.1)	

Statistical significance values are in bold.

EFS indicates event-free survival; MFC, multiparametric flow cytometry; MRD, minimal residual disease; OS, overall survival; PCR, polymerase chain reaction.

optimal number of bone marrow cells for analysis on day 15; phenotypic shift in some patients and also postinduction regeneration of normal cells can lead to inconclusive, erroneous results or difficult interpretations. It is also important to note that the samples must be analyzed shortly after collection to avoid cell death.[15–18] In our study, standardization and technical difficulties in obtaining an optimal sample might have caused such results. The Euroflow has established standard guidelines but it is evident that a more standardized approach and well-educated experienced operators are still needed to correctly evaluate the results.[19,20]

Detection of Ig/TCR gene arrangements by qPCR for MRD assessment is a more sensitive and well standardized method with consensus guidelines, but it is time consuming, labor intensive, expensive and highly complex requiring extensive knowledge and experience.[15–18] In our study, univariate analysis showed that day 33 and day 78 PCR-MRD are important prognostic factors affecting both EFS and OS. Furthermore, multivariate analysis showed day 78 PCR-MRD as the only prognostic factor affecting EFS. Our results are in accordance with the AIEOP BFM 2000 study which showed that PCR-MRD is the most relevant factor discriminating prognosis and it overshadows the importance of other known prognostic features.[7] However, this result should not be interpreted as an inferiority for MFC-MRD analysis, as this method has already shown to be effective in various studies.[6,13,14,40] ALLIC-BFM 2009 measures MFC-MRD on day 15, however, BFM-2000 measures PCR-MRD for several times and at later time points. This timing might have a role in obtaining more precise results for PCR-MRD. The latest BFM protocols both use MFC and quantitative Ig/TCR-PCR for MRD detection in order to define those who are at HR for relapse as well as early responders for better stratification of risk groups and tailoring the treatment intensity https://www.clinicaltrials.gov/ct2/show/NCT03643276?cond=AIEOP-BFM&draw=2&rank=1. In this context; it is very important to establish the infrastructure and ensure necessary standardization for both methods to make it work efficiently in our country where there is a substantial population of children with ALL.

TABLE 3. Cox Multivariate Analysis of Prognostic Factors Affecting Event-free Survival and Overall Survival

	Hazard Ratio	95% CI	*P*
Variables affecting EFS			
Day 78 PCR-MRD $\geq 10^{-3}$	8.03	2.5-25	**0.000**
Variables affecting OS			
Prednisone poor response	4.29	1-18.2	**0.049**
Day 78 PCR-MRD $\geq 10^{-3}$	5.20	1.1-23.5	**0.03**

Statistical significance values are in bold.

Parameters included in the analysis but not significant: age, prednisone poor response, day 33 PCR-MRD, initial leukocyte count.

EFS indicates event-free survival; MRD, minimal residual disease; OS, overall survival; PCR, polymerase chain reaction.

CONCLUSION

The current study showed that; 2% of patients were upstaged to HR group based on MRD results. The EFS and OS did not differ significantly between the MRD and non-MRD groups but most important factors affecting EFS was found to be PCR positivity on day 78. The establishment and standardization of both MRD methods and their implementation to current protocols are of great importance for the success of childhood leukemia treatment in our country.

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