

# Clinical Significance of Minimal Residual Disease at the End of Remission Induction Therapy in Childhood Acute Lymphoblastic Leukemia

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## Abstract

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**BACKGROUND:** Detection of minimal residual disease (MRD) in the early phase of therapy is the most powerful predictor of relapse risk in children with acute lymphoblastic leukaemia (ALL).

**AIM:** We aimed to determine the significance of MRD at the end of remission induction therapy in the prediction of treatment outcome in children with ALL.

**METHODS:** Sixty-four consecutive patients aged 1-14 years with newly diagnosed ALL were enrolled in this study from January 2010 to October 2017. All patients were treated according to the ALL IC BFM 2002 protocol. MRD was detected at the end of remission induction therapy (day 33) by multiparameter 6-colour flow cytometry performed on bone marrow specimens with a sensitivity of 0.01%.

**RESULTS:** Overall, 42.2% of patients had detectable MRD on day 33 of therapy. MRD measurements were not significantly related to presenting characteristics but were associated with a poorer blast clearance on day 8 and 15 of remission induction therapy. Patients with negative MRD status on day 33 had a 5-year event-free survival of 94.6% compared with 76.1% for those with positive MRD status ( $P = 0.044$ ).

**CONCLUSION:** MRD levels at the end of remission induction therapy measured by multiparameter flow cytometry have clinical significance in childhood ALL. High levels of MRD are strongly related to poor treatment outcome.

## Introduction

Minimal residual disease (MRD) is defined as the presence of sub-microscopic levels of leukaemic cells. [1] Detection of MRD during remission induction and consolidation therapy is the most sensitive method to evaluate treatment response and one of the strongest predictors of outcome in childhood acute lymphoblastic leukaemia (ALL). Many studies have demonstrated the prognostic significance of measuring MRD in childhood ALL, suggesting that MRD positivity at serial time points during the treatment is highly predictive of relapse, and it is associated with poor treatment outcome [2], [3], [4],

[5], [6], [7], [8], [9].

Current contemporary protocols incorporate MRD monitoring as the main stratification criterion for risk-adapted treatment. Recent studies have shown that personalised treatment based on MRD can improve the clinical outcome of children with ALL [16], [17], [18], [19], [20], [21]. Because of the strong correlation between MRD levels and risk of relapse, this concept includes treatment intensification for children with higher MRD levels and treatment de-intensification for patients with early MRD clearance. The techniques for MRD assessment allow an average detection of one leukemic cell among  $10^4$  to  $10^5$  normal cells, which represents a 100-fold increase in sensitivity compared to conventional bone marrow

cytomorphology. The most widely used methods for MRD assessment are multiparameter flow cytometric (FCM) analysis of aberrant immunophenotypes and polymerase chain reaction (PCR) amplification of different fusion genes transcript or the antigen receptor rearrangements for immunoglobulin (Ig) or the T-cell receptors (TCR). [10], [11], [12], [13], [14], [15]. Both methods are highly sensitive and specific, but expensive, complex, require qualified staff and, because of that, their use is restricted in countries with limited resources.

The purpose of our study was to determine the prognostic significance of MRD detected by flow cytometry at the end of remission induction therapy in children with ALL.

## Material and Methods

### Patients and treatment

From January 2010 to October 2017, 74 consecutive patients aged 1 to 14 years with newly diagnosed ALL were treated at the Department of Hematology and Oncology of the University Clinic for children's diseases in Skopje, Macedonia. Among the 74 patients, 64 patients in whom flow cytometric MRD assessment was done on day 33 of remission induction were enrolled in this study. Data on demographic characteristics, diagnostic immunophenotyping, molecular risk factors, early treatment response, flow cytometric MRD assay and treatment outcomes were retrospectively collected from the hospital electronic system and paper-based – medical records. The study was approved by the Ethics Committee of the Medical Faculty in Skopje.

Diagnosis of ALL was based on standard morphologic, cytochemical, immunophenotype and genetic studies. Patients were treated based on the intermediate-risk arm of ALL-IC BFM 2002 protocol consisted of induction (protocol I), consolidation (protocol M), delayed intensification (protocol II) and maintenance therapy with a total duration of 2 years. Induction chemotherapy consisted of glucocorticoids, vincristine, daunorubicin and L-asparaginase with intrathecal methotrexate. In protocol M all patients received four courses of high dose (5gr/m<sup>2</sup>) methotrexate. Eight children in our cohort (2 children with BCR-ABL positive ALL and 6 with high positive levels of MRD at the end of induction therapy) were allocated into a high-risk group, and they were treated according to the high-risk arm of ALL – IC BFM 2002 protocol. One of the 2 patients with BCR-ABL positive ALL was treated with intensive chemotherapy alone, whereas the second patient was treated with chemotherapy plus imatinib. Informed consent had been obtained for all patients from their guardians before initiation of chemotherapy following The

Declaration of Helsinki.

In Macedonia flow cytometry, which is performed at the Clinic of Hematology, is used in diagnosis for acute leukaemia, but its applicability in MRD assessment is limited. MRD analysis at the end of induction therapy (day 33) was performed in the reference flow cytometric laboratory of the General Hospital George Papanikolaou in Thessaloniki, Republic of Greece by multiparameter 6 color flow cytometry using bone marrow mononucleated cells which were sent immediately after bone marrow aspiration (sternum puncture) collected in ethylenediamine tetra-acetic acid tube. Leukaemia-associated immunophenotypes were investigated with various combinations of monoclonal antibodies conjugated to the following fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), phycoerythrin-cyanin 5.1 (PC5), phycoerythrin-cyanine 7 (PE Cy7) and *allophycocyanin-cyanine 7* (APC-Cy7), (Table 1 and 2).

**Table 1: Monoclonal antibody combinations used for MRD detection in precursor B ALL**

| FITC | PE    | APC  | PC5  | PECy7 | APC-Cy7 |
|------|-------|------|------|-------|---------|
| CD58 | CD38  | CD10 | CD45 | CD34  | CD19    |
| CD24 | CD38  | CD10 | CD45 | CD34  | CD19    |
| CD38 | CD22  | CD10 | CD45 | CD34  | CD19    |
| CD81 | CD20  | CD10 | CD45 | CD34  | CD19    |
| CD38 | CD200 | CD10 | CD45 | CD34  | CD19    |

Cell staining was performed on FACSCanto II flow cytometer, using the FACSDiva software (BD Biosciences) for analysis. This detection method allows the identification of one leukemic cell among 10 000 or more normal bone marrow cells. MRD positivity was defined as  $\geq 0.01\%$  of mononuclear cells expressing leukaemia-specific immunophenotypes [3].

**Table 2: Monoclonal antibody combinations used for MRD detection in T cell ALL**

| FITC | PE   | APC | PC5 | PECy7 | APC-Cy7 |
|------|------|-----|-----|-------|---------|
| CD7  | CD1a | CD3 | CD5 | CD34  | CD45    |
| CD38 | CD1a | CD3 | CD5 | CD34  | CD45    |
| CD99 | CD1a | CD3 | CD5 | CD34  | CD45    |

### Statistical analysis

October 31, 2018, was chosen as the reference date for the collection of data. The mean observation time was 45.7 months (range 1-100 months). Associations between MRD, presenting features, and early treatment response was analysed with the  $\chi^2$  test or Fisher exact test. Event-free survival (EFS) was calculated from the date of diagnosis to date of first event or date of last follow up if no event occurred. The event was resistance to therapy, relapse or death from any cause. EFS and survival curves were estimated according to Kaplan-Meier and groups were compared by log-rank test. The significance level of 0.05 was used in all statistical test. All statistical analyses were performed using SPSS (Statistical Package for the Social Science) version 23.0.

## Results

### Patients' characteristics

Presenting clinical features of the 64 patients were summarised in Table 3. The median age of patients was 5.6 years (range 1-14 years). There was a slight predominance of males (57.8%). The median WBC count at presentation was  $32.07 \times 10^9/L$  (range  $0.89-194.3 \times 10^9/L$ ). Precursor B cell ALL was diagnosed in 52 (81.2%) patients and T cell ALL in 12 (18.8%). CNS involvement at diagnoses was confirmed in 3 (4.7%) patients. The majority of patients were considered as standard risk based on NCI criteria. BCR-ABL was documented in 2 out of 45 patients.

### Relation among MRD levels, presenting features, and early treatment response

Of the 64 patients, 37 (57.8%) had a bone marrow that was negative for MRD ( $< 0.01\%$ ), whereas 27 (42.2%) were MRD positive ( $\geq 0.01\%$ ) at the end of remission induction therapy. Among patients with MRD positive status, the levels of MRD were  $0.01- \leq 0.1\%$  in 11 (40.8%) children,  $0.1- \leq 1\%$  in 12 (44.4%) patients and  $>1\%$  in 4 (14.8%) patients. Regarding MRD distribution by immunophenotype, we observed a higher rate of MRD positive findings among patients with precursor B cell ALL compared to patients with T cell ALL (44.2% versus 33.3% respectively), but it should be noted that quantitative MRD levels were higher in T cell ALL.

**Table 3: MRD distribution according to patients' clinicobiological features**

| Characteristics               | Total     | MRD level on day 33 |               | P     |
|-------------------------------|-----------|---------------------|---------------|-------|
|                               |           | $< 0.01\%$          | $\geq 0.01\%$ |       |
|                               | 64 (%)    | 37 (%)              | 27 (%)        |       |
| Gender                        |           |                     |               | 0.523 |
| male                          | 37 (57.8) | 21 (56.8)           | 16 (59.3)     |       |
| female                        | 27 (42.2) | 16 (43.2)           | 11 (40.7)     |       |
| Age                           |           |                     |               | 0.194 |
| 1 to < 10                     | 56 (87.5) | 34 (91.9)           | 22 (81.5)     |       |
| 10 -14                        | 8 (12.5)  | 3 (8.1)             | 5 (18.5)      |       |
| WBC count ( $\times 10^9/L$ ) |           |                     |               | 0.615 |
| $\leq 50 \times 10^9/L$       | 52 (81.2) | 30 (81.1)           | 22 (81.5)     |       |
| $> 50 \times 10^9/L$          | 12 (18.8) | 7 (18.9)            | 5 (18.5)      |       |
| Immunophenotype               |           |                     |               | 0.362 |
| Precursor B-ALL               | 52 (81.2) | 29 (78.4)           | 23 (85.2)     |       |
| T cell-ALL                    | 12 (18.8) | 8 (21.6)            | 4 (14.8)      |       |
| CNS involvement               |           |                     |               | 0.329 |
| present                       | 3 (4.7)   | 2 (5.4)             | 1 (3.7)       |       |
| absent                        | 61 (95.3) | 35 (94.6)           | 26 (96.3)     |       |
| NCI risk group                |           |                     |               | 0.151 |
| standard                      | 48 (75.0) | 29 (78.4)           | 19 (70.4)     |       |
| high                          | 16 (25.0) | 8 (21.6)            | 8 (29.6)      |       |
| BCR-ABL                       |           |                     |               | 0.019 |
| positive                      | 2 (3.1)   | 0 (0)               | 2 (7.4)       |       |
| negative                      | 43 (67.2) | 24 (64.9)           | 19 (70.4)     |       |
| unknown                       | 19 (29.7) | 13 (35.1)           | 6 (22.2)      |       |
| Prednisone response           |           |                     |               | 0.003 |
| PPR                           | 7 (10.9)  | 1 (2.7)             | 6 (22.2)      |       |
| PGR                           | 57 (89.1) | 36 (97.3)           | 21 (77.8)     |       |
| BM - 15 day                   |           |                     |               |       |
| M1                            | 45 (70.3) | 32 (86.5)           | 13 (48.2)     |       |
| M2                            | 14 (21.9) | 4 (10.8)            | 10 (37.0)     |       |
| M3                            | 5 (7.8)   | 1 (2.7)             | 4 (14.8)      |       |

Abbreviations: WBC = white blood cells; CNS = central nervous system; NCI = National Cancer Institute; NCI standard risk group = age 1 to <10 years, and WBC  $< 50 \times 10^9/L$ ; NCI high risk group = age  $\geq 10$  years or WBC  $> 50 \times 10^9/L$ ; PPR = absolute blast count in the peripheral blood  $\geq 1 \times 10^9/L$ ; PGR = absolute blast count in the peripheral blood less than  $1 \times 10^9/L$  after 7 days of prednisone and one dose of intrathecal methotrexate on day 1; M1 BM (bone marrow) =  $< 5\%$ , M2 = 5 to 25% and M3 =  $\geq 25\%$  leukemic blasts in BM.

Table 3 shows the relation between levels of MRD on day 33 and the clinicobiological features of the disease. In our analysis, the presenting features including gender, age, WBC count at diagnoses, CNS involvement, immunophenotype, NCI risk status and molecular risk factors did not differ significantly between patients with negative and positive MRD status at the end of induction therapy. Two cases with BCR-ABL positive ALL, which is prognostically unfavourable had  $\geq 0.1\%$  leukemic cells at the end of remission induction, but this failed to reach statistical significance due to small sample size ( $P = 0.151$ ). These findings indicate that presenting prognostic features could not identify patients who will have undetectable MRD at the end of remission induction therapy. We also tested whether earlier treatment response determined by prednisone response and bone marrow morphology on day 15 would predict the presence of MRD after completion of induction therapy. In our series, MRD levels differed significantly between prednisone poor and good responders. Patients with prednisone poor response (PPR) were more likely to be MRD positive at day 33 than patients with prednisone good response (PGR) ( $P = 0.019$ ). Bone marrow morphology on day 15 also had a significant impact of MRD status on day 33. Patients with M2 and M3 bone marrow morphology on day 15 were more likely to be MRD positive at the end of induction therapy than patients with M1 ( $P = 0.003$ ). In contrast to presenting clinical features, our findings indicate that the earlier clearance of leukemic blasts in peripheral blood and bone marrow could predict patients who will attain negative MRD status at the end of remission induction therapy.

When comparing 2 groups of patients based on end-induction MRD status, 27 MRD positive patients on day 33 had a significantly lower 5-year EFS than 37 MRD negative patients (76.1% versus 94.6; respectively,  $P = 0.044$ ; Figure 1). The main event in our series that contributed to the EFS was the occurrence of relapse. Relapses were recorded in 8 (12.5%) out of a total of 64 patients. Among patients with positive MRD findings, the relapse rate was 22.2% compared to 5.4% among MRD negative patients (Table 4). Of note, none of the MRD negative patients with precursor B cell ALL experienced relapse during the follow-up study period. Thus, relatively rapid elimination of MRD in patients with this subtype of leukaemia identifies cases with an excellent prognosis.

**Table 4: Description of events contributing to EFS comparing MRD negative and positive patients**

| Events                    | MRD negative patients | MRD positive patients |
|---------------------------|-----------------------|-----------------------|
|                           | N = 37                | N = 27                |
|                           | N (%)                 | N (%)                 |
| Isolated BM relapse       | 2 (5.4)               | 5 (18.5)              |
| Combined relapse (BM+CNS) | 0                     | 1 (3.7)               |
| Death in remission        | 0                     | 2 (7.4)               |
| Total deaths              | 2 (5.4)               | 7 (25.9)              |

## Discussion

The prognostic importance of MRD in childhood ALL is well established in numerous clinical studies [2], [3], [4], [5], [6], [7], [8], [9], [10], [11], [12], [13], [14], [15]. Modern treatment protocols for childhood ALL recommended MRD monitoring at multiple time points to evaluate the effectiveness of chemotherapy in the elimination of leukemic cells. Complex and sophisticated methods such as PCR-based techniques or multiparametric flow cytometry have been developed and evaluated to detect MRD. MRD measurement during the early phase of treatment (on day 8 and day 15/19) and at the end of induction therapy (day 29, 33 or 42) is considered the main predictor of treatment outcome and essential tool for risk stratification, aimed at both treatment intensification and reduction [8], [9], [10], [11], [12], [13], [14], [15], [23], [24], [25].

Patients with less than 0.01% leukemic cells at the end of remission induction are likely to have an excellent treatment outcome, whereas patients with high levels (i.e.,  $\geq 1\%$ ) of MRD at the end of the induction phase have a significantly higher risk of relapse and should be considered for alternative treatments. [3,4,8] Recent studies have demonstrated that intensification of therapy for patients with high levels of MRD can improve their outcome [12], [15].

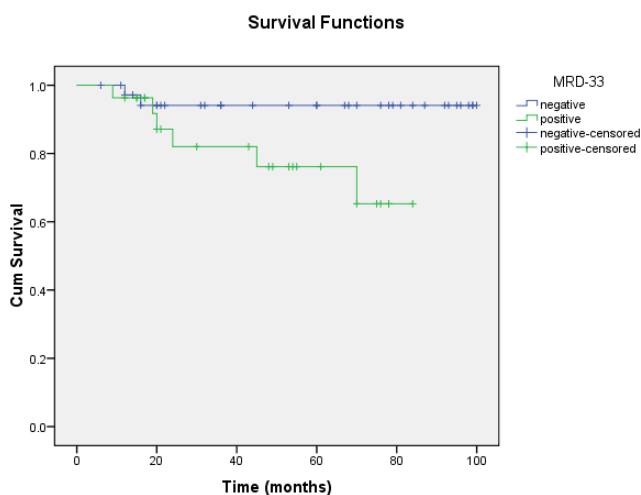


Figure 1: Kaplan-Meier estimate of 5-year Event-free survival based on MRD status on day 33 (negative versus positive) EFS = 94.6% versus 76.1% respectively,  $P = 0.044$

In this study, MRD measurement was performed at the end of induction therapy, as this time point seemed most relevant for the treatment decision. Our data defines a cohort of 37 patients with negative MRD status at the end of induction therapy who have a superior EFS compared to those with positive MRD and this finding is consistent with numerous clinical studies [2], [3], [5], [6], [7], [8], [10], [12], [13], [14], [15], [23]. Researchers of Children's Oncology Group (COG) studied the prognostic impact

of MRD measured by flow cytometry in the peripheral blood at day 8, end-induction (day 29) and end-consolidation bone marrows in 2143 children with precursor B-cell ALL. The presence of MRD in day-8 blood and day-29 bone marrow was associated with a lower EFS in all risk groups; even patients with 0.01% to 0.1% day-29 MRD had poor outcome compared to MRD negative patients at the end of remission induction (5-year EFS 59% vs 88%) [8]. In the AIEOP – BFM ALL 2000 study, 3184 patients with precursor B cell ALL were stratified by MRD measured on days 33 and 78 based on immunoglobulin and TCR gene rearrangements into three groups with a significantly different outcome. Patients defined as standard risk (42%) showed a 5-year EFS estimated at 92.3%, while intermediate (52%) and high-risk patients (6%) showed a 5-year EFS of 77.6% and 50.1%, respectively [10]. In the study by Basso et al. measurement of MRD by flow cytometry on day 15 bone marrow was the most powerful early predictor of relapse. Standard risk patients had a significantly lower 5-year cumulative incidence of relapse compared to patients from the intermediate and high-risk group (7.5% vs 17.5 vs 47.2%, respectively) [9].

In our cohort, certain clinicobiological features of ALL didn't show relation to the speed of leukemic blasts reduction. Investigators of Total Therapy studies XIII A and XIII B at St. Jude Children's Research Center have shown that the patient's age and the presence or absence of adverse genetic abnormalities were directly related to the speed and extent of initial cyto-reduction [3]. And other study groups have observed slow clearance of MRD in NCI high-risk children, or with leukemic blasts expressing BCR-ABL [2], [26]. In our cohort, the BCR-ABL fusion transcript was confirmed in two patients, and both of them were MRD positive at high levels at the end of induction therapy. Despite the lack of statistical significance, probably due to the small sample size, it can be said that our results are in agreement with the previous studies [2], [3], [26]. MRD clearance is depended by the biology of the leukemic blasts, but other factors as well including specific host germline pharmacogenetics polymorphisms can affect in vivo treatment response and regulate treatment efficacy in each patient [27].

Prednisone response and bone marrow morphologic evaluation on day 15 are an integral part of the BFM protocol's stratification scheme. Prediction of treatment outcome by conventional morphological assessment of treatment response is still of great importance, especially to countries with limited resources for MRD monitoring [28]. Our study confirmed a significant association between rapid early clearance of leukemic cells from peripheral blood and bone marrow and attaining MRD negative status at the end of induction therapy. This is in agreement with the previous study of Fronkova et al., who observed that in patients treated according to ALL IC BFM 2002 protocol, MRD negativity at day 33

was associated with good prednisone response and non – M3 morphology at day 15 [29]. Researching whether it is possible to avoid MRD testing in some subgroups of ALL patients, Fronkova et al., found that morphological criteria in ALL-IC are able to identify most MRD high-risk patients, but fail to define the MRD low-risk group, for who is possible treatment reduction to avoid long-term toxicities, which is the challenge of modern leukaemia treatment [29].

In conclusion, measurement of blast clearance allowing the quantitative definition of MRD is a mandatory tool for favourable treatment outcome of childhood ALL. Our study confirmed the excellent outcome for childhood ALL in patients with negative MRD monitoring by flow cytometry at the end of induction therapy. MRD assessment is still not available in our treatment centre and considering its prognostic importance; there is an urgent clinical need to introduce in routine practice.

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