

Study of Minimal Residual Disease in Adults with B-Lineage Acute Lymphoblastic Leukemia by Flowcytometry

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Abstract

Background: After achieving morphological remission, existence of few number of leukemic cells in the patient's blood represents the minimal residual disease (MRD) and its monitoring helps in evaluating early treatment response and future relapse. **Patients and methods:** Eighty seven newly diagnosed (B-ALL) cases were enrolled in the present study in the time period from October 2013 to October 2016. A panel of 4 monoclonal antibodies (CD10FITC, CD19PE, CD34PercP and CD45APC) were defined at diagnosis and after morphological remission for tracing of minimal residual disease (MRD). **Results:** Eighty seven newly diagnosed B-ALL cases were included in the present study of which 73 (84%) showed positive expression to CD45 in combination with (CD10, CD19 and CD34) at diagnosis, which allow us to use this combination for further assessment of MRD after morphological remission. In our study 65% of patients had negative MRD (<0.01), while 35% of patients had positive MRD (≥ 0.01). The DFS and OS for patients with MRD-ve were significantly higher than those with MRD + ve ($P = 0.01$ & $P = 0.04$) respectively. **Conclusion:** MRD detection by flow cytometry using the combination of CD45 with CD10, CD19 & CD34 is an easy and reliable method. Patients with positive MRD are at higher risk of relapse and have inferior overall survival rates compared to those with MRD-ve. Future studies focusing on treatment intensification for the group of patients with +ve MRD aiming to improve the treatment outcome are warranted.

Keywords

Acute Lymphoblastic Leukemia (ALL), Minimal Residual Disease (MRD), Flowcytometry (FCM), Complete Response (CR), Disease Free Survival (DFS), Overall Survival (OS)

1. Introduction

Minimal residual disease describes the leukemic cells that remain in the blood and cannot be detected after morphological remission. Its monitoring has become routine clinical practice nearly in all children acute lymphoblastic leukemia (ALL) treatment and in many adult ALL patients [1].

According to the 2008-2011 National Cancer Registry Program, the incidence of lymphoid leukemia in patients aged 18 years or more was 33.7/100,000 men and 20.2/100,000 women [2].

Monitoring of minimal residual disease (MRD) in patients with ALL offers a way in assessing early treatment response and relapse detection. MRD measurement by flowcytometry depends on the detection of leukemia associated immunophenotypes, which can be used to differentiate them from normal hematopoietic cells [3].

The main principle underlying all MRD assays is that the process of leukemogenesis has resulted in both molecular and cellular changes that distinguish leukemic cells from their normal counterparts, so these leukemia-associated features are identified at diagnosis or at relapse and then used to monitor MRD. Consequently, techniques for MRD detection can be classified based on the type of cell marker used to identify the malignant cell clone. MRD measurements can detect the effect of the novel treatment and can be used as a surrogate end point. Identification of new markers in leukemia and the use of highly specific assays should facilitate routine monitoring of MRD and so help in understanding the cellular and biologic features of leukemic cells that resist chemotherapy *in vivo* [4].

Selection of treatment intensity and duration can depend on results of MRD researches and estimation of the optimal time for hematopoietic stem cell transplantation [5].

2. Aim of the Study

The aim of this study is to determine the value of MRD monitoring by FCM in adult B-ALL patients after achieving morphological remission and its impact on overall & disease free survival.

3. Subjects and Methods

The present study was carried out at Medical Oncology & Hematology Department and Clinical Pathology Department of Zagazig University Hospitals in the time period from October 2013 and October 2016.

3.1. Subject

Eighty seven newly diagnosed B-ALL cases were enrolled in the present study of which 73 (84%) showed positive expression to CD45 in combination with (CD10, CD19 and CD34). They were 54 males and 19 females with a male/female ratio of 2.8:1. The median age of the studied patients was 29 years ranging from 15 to

54 years. All patients received hyper CVAD regimen.

All participants were informed adequately about the aim of the study and consented to donate samples for research purpose. Samples were obtained after informed consents and in accordance with the procedures approved by the human ethics committee.

Eligibility criteria:

- Denovo B-ALL
- Philadelphia–ve
- Age >15 years
- No significant chronic illness
- No previous malignancy
- No previous chemotherapy

Exclusion criteria:

- Relapsed or refractory ALL
- T-ALL
- Philadelphia + ve
- Previously diagnosed with cancer and/or received chemotherapy
- Significant co-morbidity e.g. heart failure, renal insufficiency, chronic liver disease

3.2. Methods

All patients were subjected to thorough history taking, full clinical examination, complete blood count (Sysmex XT1800) with examination of a Leishman-stained smear, bone marrow (BM) aspiration for morphology and cytochemistry. Liver and kidney functions tests, uric acid level and lactate dehydrogenase (Dimension R-XL max), serum electrolytes (Cobas Integra 400 plus). Cerebrospinal fluid (CSF) examination was also done in addition to cytogenetics for Philadelphia chromosome. Chest radiographs, abdominal ultrasound and other radiological examinations.

Immunophenotyping of blast cells using the whole blood lysing technique was performed using a panel of monoclonal antibodies directly conjugated with FITC, PE, PerCP and APC, were used; the monoclonal antibodies included CD3, CD5, CD7, CD10, CD13, CD19, CD20, CD22, CD33, CD34, CD45, CD79a, TDT, HLA-DR, and MPO, and appropriate Isotypic controls were used in all cases. All monoclonal antibodies were purchased from Becton–Dickinson BD (San Diego, California, USA). Cells were considered positive for any monoclonal antibodies when >20% of the cells express this marker except for TDT, MPO and CD34, the cutoff value is 10% only. Diagnosis of B-lineage acute lymphoblastic leukemia was based on morphological, cytochemical and immunophenotypic characteristics of leukemic blasts. The combination CD10 FITC, CD19PE, CD34 PercP and CD45APC in one tube was used for the detection of MRD. This combination had been defined at diagnosis and post induction after morphological remission for tracing of (MRD) as this combination was one of the highest incidence marker expression and it was beneficial in differentiating hematogones

from residual blasts in ALL cases after induction. The rational for MRD detection is to use sequential gating.

In all cases, tight lympho-population gate was applied on SSC vs. FSC then CD45 vs. SSC and CD19 co-expressing CD34 population, then subsequently gating on CD34 and CD45. Leukemic events were defined at dot plot in a region with estimated number of events from statistics, distinguishing of leukemic cells from their normal counterparts. In cases of B-ALL cells, the normal equivalent cells are the B cells progenitors (hematogones), which normally reside in the bone marrow and can also be found in low proportion in peripheral blood, so the challenge is to differentiate these normal progenitors from the malignant cells [6].

We used CD45 mean fluorescence intensity (MFI) to differentiate between the blasts, which are dim for CD45 and the lymphocytes, which are bright for CD45. Gates were performed to detect the position of the blasts from the time of diagnosis, and we chose the site of CD45 expression which is dim or moderate and not high. Then on doing follow up after morphological remission comparison was done on the same chosen gate for blast detection away from position of proliferated lymphocytes (hematogones) with high expression of CD45 as shown in **Figure 1**.

Minimum target sensitivity for quantification of MRD was defined as (0.01%). Cut off point of MRD was $<10^{-4}$ (0.01%). MRD low risk for patients with MRD $<10^{-4}$ at all examined time points after induction and MRD high risk for patients with MRD $>10^{-4}$ at any time-point [7] [8].

3.3. Treatment Plan

Four cycles of *hyper-CVAD* alternating with 4 cycles of *high dose methotrexate and cytarabine* were given every 21 days as follows:

Hyper-CVAD regimen:

Cyclophosphamide: 300 mg /m² IV every 12 hours for 6 doses on days 1 - 3

Mesna: 600 mg IV over 24 hours on days 1 - 3 ending 6 hours after the last dose of cyclophosphamide

Vincristine: 2 mg IV on days 4 and 11

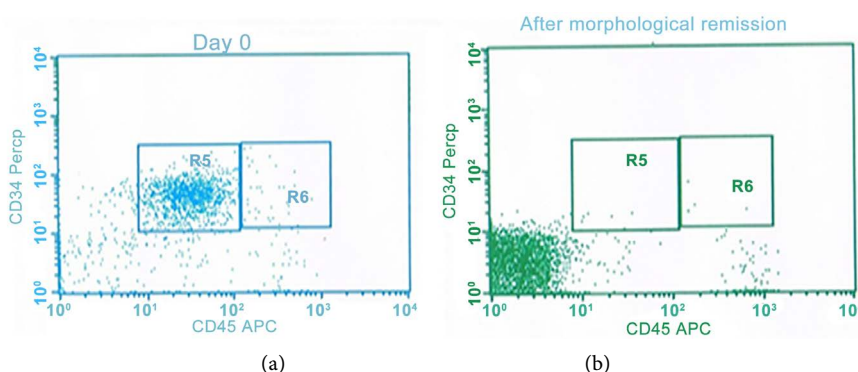


Figure 1. Comparison between blast events & MFI of CD45 at day (0) and after morphological remission for the same B-ALL patient.

Doxorubicin: 50 mg/m² IV on day 4

Dexamethasone: 40 mg IV on days 1 - 4 and 11 - 14

High dose methotrexate and cytarabine:

Methotrexate 200 mg/m² IV over 2 hours, followed by 800 mg/m² IV over 24 hours on day 1

Leucovorin: 15 mg IV every 6 hours for 8 doses, starting 24 hours after the completion of methotrexate infusion

Cytarabine: 2000 mg/m² IV every 12 hours for 4 doses on days 2 - 3

Methyl prednisolone: 50 mg IV bid days 1 - 3

CNS prophylaxis: repeated with every chemotherapy cycle “depending on risk of CNS disease”

Methotrexate: 12 mg IT on day 2

Cytarabine: 100 mg IT on day 8

Supportive care

Ciprofloxacin: 500 mg PO bid

Fluconazole: 200 mg IV OD

Acyclovir: 200 mg PO bid

G-CSF: 10 ug/kg/day SC starting 24 hours after the end of chemotherapy

3.4. Statistical Analysis

The statistical package for the social sciences (SPSS software 19; SPSS Inc., Chicago, USA) was used for data analysis [9]. Survival analysis was done using the Kaplan Meier method and the Log Rank test was used to compare survival curves. Correlation between quantitative variables was done by the r-test. Significance level of 0.05 was used in all statistical tests.

4. Results

Eighty seven adult B-ALL patients were enrolled in the study according to the eligibility criteria, only 73 (84%) of them showed positive expression to CD45 in combination with (CD10, CD19 and CD34) and were included in our research. They were 54 males and 19 females. Median age was 29 years ranging from (15-54 years). Patients' characteristics at diagnosis are shown in **Table 1**.

The median total leucocytic count (TLC) was $10.7 \times 10^3/\text{mm}^3$ with a range of $0.9 - 152.5 \times 10^3/\text{mm}^3$. CNS leukemia infiltration was present in 6.8% (5/73) of patients. Laboratory characteristics of the patients are shown in **Table 2**.

Response to Induction Chemotherapy 54 (74%) of patients achieved morphologic remission, while 19 (26%) failed to achieve remission. Patients achieving morphologic remission were evaluated for MRD using flowcytometric analysis. MRD positivity was found in 19 cases (35%), while 35 patients (65%) were MRD-ve.

Table 3 clarifies the correlation between blast events (CD34⁺/CD45⁺) & MFI of CD45. It shows that there was a negative correlation between No. of blast events & MFI of CD45, where the decrease in blast events is associated with increase in MFI, but it is statistically not significant.

Table 1. Clinical Patients characteristics.

Item	No (%)	Item	No (%)
Age(year)		Lymphadenopathy	
Range	15 - 54	Present	23 (31.5)
Median	29	Absent	50 (68.5)
Sex		Splenomegaly	
Female	19 (26%)	Present	43 (59)
Male	54 (74%)	Absent	30 (41)
Fever		Hepatomegaly	
Present	40 (54.8)	Present	20 (27.4)
Absent	33 (45.2)	Absent	53 (72.6)
Pallor		Purpura	
Present	45 (61.6)	Present	14 (19.2)
Absent	28 (38.4)	Absent	59 (80.8)
		Response to chemotherapy	
CNS manifestation	4 (5.4%)	CR	54 (74%)
		No CR	19 (26%)

Table 2. Laboratory Patients characteristics.

Item	No (%)	Item	No (%)
Hb (g/dl)		ESR ml/h	
Range	6.9 - 13.6	Range	25 - 145
Median	11.8	Median	61
Classification		Peripheral blood blasts %	
<10	48 (65.8%)	Range	15 - 85
>10	25 (34.2%)	Median	42
WBCs $\times 10^3 / \text{mm}^3$		Bone marrow blasts%	
Range	0.9 - 152.5	Range	41 - 94
Median	10.7	Median	64
Classification		BM morphology	
<50,000	60 (82.2%)	L1	34 (46.6%)
>50,000	13 (17.8%)	L2	28 (38.4%)
Platelets $\times 10^3 / \text{mm}^3$		L3	11 (15%)
Range	8 - 197	Immunophenotyping	
Median	33	Pro B-ALL	11 (15.1%)
Classification		C-ALL	42 (57.5%)
<100,000	55(75.4%)	Pre B-ALL	12(16.4%)
>100,000	18(24.6%)	Mature B-ALL	8 (11%)
Initial CSF examination		LDH U/L	
Positive for blast cells	5 (6.8%)	Range	220 - 9900
Negative for blast cells	68 (93.2%)	Median	880
MRD (54 patients in CR)			
+ve cases	19 (35%)		
-ve cases	35 (65%)		

Disease Free Survival:

The 2-year DFS for the studied patients was 43%. The median DFS (95%CI) was 22 months (14.2 - 29.7). The 2-year DFS was significantly higher in patients

with MRD–ve (58%) compared to (14%) for patients with MRD +ve (Log Rank Test; $P = 0.01$) as shown in **Table 4**.

Overall Survival:

The 2-year OS for the studied patients was 46%. The median OS (95% CI) was 26.1 months (23.5 - 29.2). The 2-year OS was significantly higher in patients with MRD-ve (58%) compared to (22%) for those with MRD +ve (Log Rank Test; $P = 0.04$) as shown in **Table 4**.

Figure 1 represents the comparison between blast events & MFI of CD45 at day (0) and after morphological remission for the same B-ALL patient. The dot-plot analysis of flowcytometry (a) shows that the region gate R5 represent CD34positive blasts with dim expression of CD45 (MFI 23.75) and region gate R6 represent Hematogones (CD34 positive with high expression of CD45) at day (0); (b) shows that there's a marked decrease in blast number in region gate R5 which represent MRD, MFI of CD45 (9.9), MRD = 0.06.

5. Discussion

MRD is attributed to the few number of cells remaining in the patient's blood during treatment or during the remission period and is the main reason for relapse in leukemic patients. Molecular methods are used nowadays as a golden standard, where FCM for MRD determination is getting developed day-by-day.

Detection of MRD is very important in detecting the efficacy of treatment and the patient's remission status and expecting the possibility of relapse. MRD diagnosis also helps in assigning different treatment regime ranging from significant treatment reduction to mild or strong intensification. In addition to its great role in relapsed patients and that undergoing stem cell transplantation as a guide to treatment strategy.

Table 3. Correlation between blast events (CD34+/CD45+) & MFI of CD45.

	MFI of blasts with CD45 ⁺	
	R	P
Percent of blasts CD45 ⁺ /CD34 ⁺	-0.128	>0.05

$P > 0.05$ is not significant.

Table 4. Comparison between DFS & OS in both MRD+ve and MRD–ve Patients.

	All Patients	MRD–ve	MRD+ve	P
DFS (month)				
Mean (CI)	21.7 (18.2 - 25.2)	25.6 (21.7 - 29.5)	13.1 (9.3 - 16.8)	0.01
Median (CI)	22 (14.2 - 29.7)	Not reached	12 (6.7 - 17.3)	
OS (month)				
Mean (CI)	25.6 (22.4 - 28.8)	27.7 (24.2 - 31.3)	20.5 (15.1 - 25.8)	0.04
Median (CI)	26.1 (23.5 - 29.2)	Not reached	20 (13.3 - 26.7)	

The outcome of ALL in adults is challenging as compared with that observed in children. The rate of disease relapse is much higher in adults. Although cure rates in children approach 90%, no more than 40% of adult patients remain free of leukemia after 5 years, and this rate is much lower in older patients [10].

The CR rate in our study was 74% compared to 88% in a study done in NCI [11], however their study included patients with both B and T-ALL, while our patients were B-ALL as shown in **Table 1**.

Measurement of MRD might be used as an end point that significantly shortens the follow up period. MRD has been shown to be prognostic essentially at every time point studied, though the most useful measurements appear to be relatively early in therapy, during or after induction and early in consolidation [12].

There are cut-off points for MRD, where $>0.01\%$ blasts in bone marrow at any time points during treatment had a significantly increase risk to relapse [13]. MRD $\geq 1\%$ at the end of induction remission therapy and MRD $\geq 0.1\%$ during continuation therapy are certainly at high risk of relapse. On the other hand MRD can help in identifying patients with favorable outcome [13].

Many cutoff values were applied by adult ALL study groups, which differ from one another in patients population and the MRD time point. NILG takes cutoff value of 1×10^{-4} at week 16 and absence of detectable MRD at week 22 [14]. PETHEMA takes a cut off value of 5×10^{-4} at weeks 16 to 18.7. Another Group for Research on Adult Acute Lymphoblastic Leukemia takes a cutoff value of 1×10^{-4} at week 6 for all ALL Philadelphia negative patients [15], while a cutoff 10^{-3} for high-risk patients [12]. However, all these studies highlight the prognostic value of MRD detection in adult patients with ALL. In our study we performed flow cytometric analysis at day (O) to be followed by MRD flowcytometric detection to patients in CR (Complete Response) after achieving morphological remission.

In our study, the results of minimal residual disease status of B-ALL patients after morphological remission showed that (65%) of patients had negative MRD (<0.01), 35% of patients had positive MRD (≥ 0.01) as shown in **Table 2**.

CD45 in combination with CD10, CD19 and CD34 were positive in 84% of our patients at diagnosis, which allow us to use this combination for further assessment of MRD after morphological remission, Campana and Coustan Smith, 2003 [16], found the combination which includes (CD19, CD34, CD10, CD45) to be informative in 30% - 50% of cases.

Lucio *et al.*, (2001) [17] reported that CD45, CD19 and CD34 are informative in 22.2% of B-ALL, whereas Vidriales *et al.*, (2003) [17] stated that CD45, CD10, CD19, and anti-TdT combination was applicable to 70% - 80% of B-ALL. Patkar *et al.*, (2012) [18] found that CD20, CD10, CD45 and CD19 combination was informative in 71.4% of B-ALL cases.

The cumulative DFS at 2 years for our study group was 43% in comparison to 42% DFS at 2 years in NCI [11], and 64% & 53% reported by others [19] & [20], while the cumulative OS at 2 years for our study group was 46% in comparison

to 49% in NCI [11] and 43% at 3-years follow up reported by Larson *et al.* [21]. Our data are shown in **Figures 2-5**.

It is accepted that cluster of MRD cells between 10 and 100 have to be identified in a given sample to assure that MRD cells have been seen. This is for achieving sensitivity between 10^{-4} to 10^{-5} , consequently 10^5 to 10^6 leukocytes have to be screened. In a study done by [22] they stated that the levels of MRD are proportional to the risk of relapse, MRD equal or greater than 1% at the end

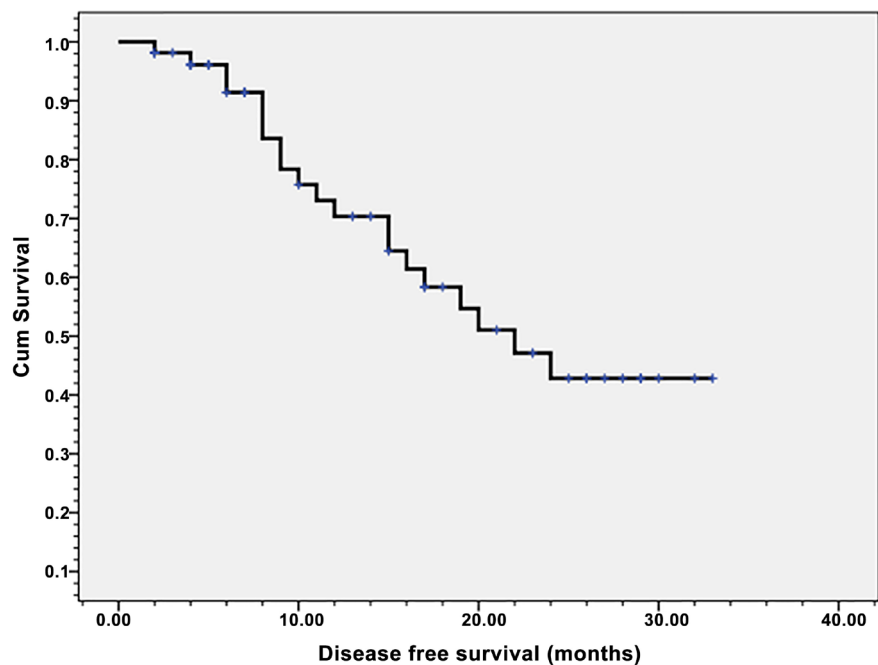


Figure 2. Disease free survival for all patients.

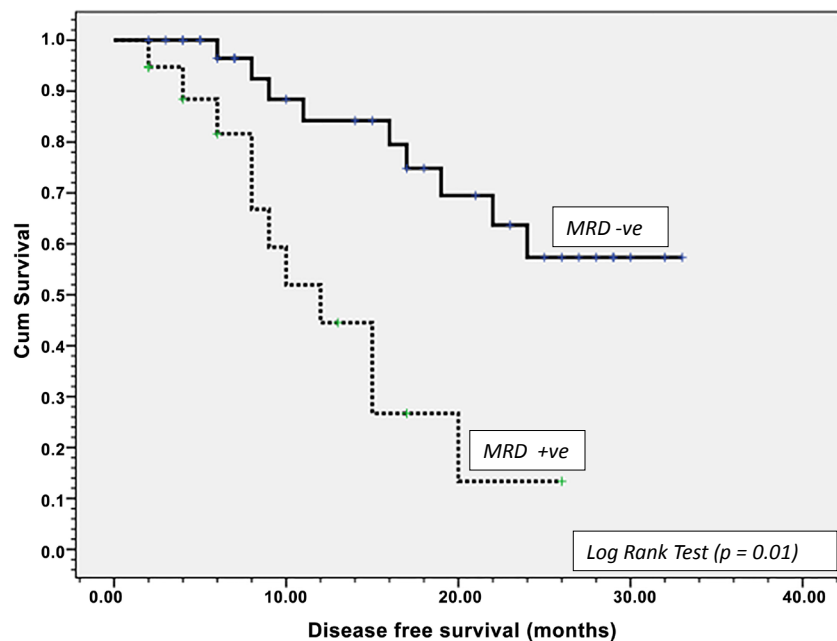


Figure 3. Disease free survival according to MRD status.

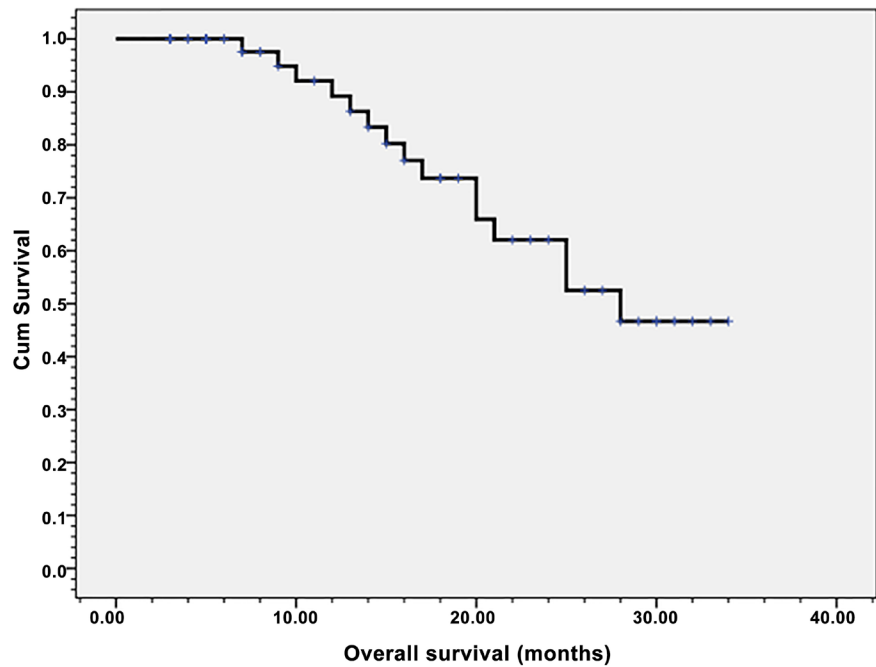


Figure 4. Overall survival for all patients.

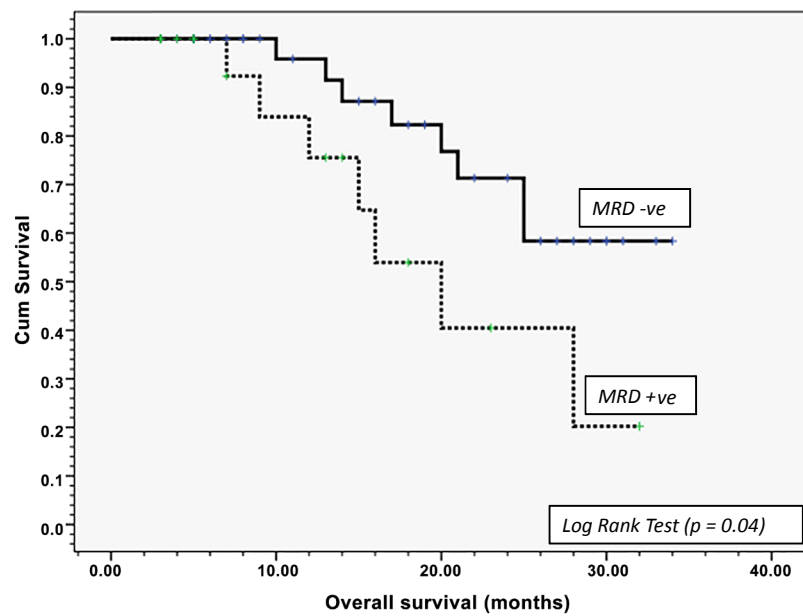


Figure 5. Overall survival according to MRD status.

of induction remission therapy is accompanied with unfavorable outcome, so recommending transplantation in first remission for these patients. The International Berline-Frankfurt-Munster (I-BFM) Study Group found that patients with MRD levels of 0.1% or higher on both day 33 and day 78 of treatment had a relapse rate of 75%, prompting treatment intensification for this group of patients is needed [23].

Patients with early clearance of leukemic cells typically remain MRD-negative, and their prognosis is excellent with current treatment protocols [24]. Some of the studies stated that treatment deintensification should be considered for these patients, others were against this and stated that early MRD negativity might be a good prognostic feature only in the context of intensive therapy. Thus, if therapy is deintensified, the risk of relapse of MRD-negative patients might increase significantly, we agree with the second opinion, however deintensification might be especially useful when intensive therapy confers a high risk of serious toxicities or in older adult patients, where the potential benefits of treatment deintensification might outweigh the risk of relapse.

6. Conclusion

MRD detection by flow cytometry using the combination of CD45 with CD10, CD19 & CD34 is an easy and reliable method. Patients with positive MRD are at higher risk of relapse and have lower overall survival rates compared to those with MRD-ve. Future studies focusing on treatment intensification for the group of patients with +ve MRD aiming to improve the treatment outcome are warranted.

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