RESEARCH ARTICLE

Impact of Minimal Residual Disease Detection by Next Generation Flow Cytometry on Outcome of Egyptian Patients with Acute Lymphoblastic Leukemia

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Abstract

  Introduction: Recently, the identification of minimal residual disease (MRD) that persists after chemotherapy has emerged as the most powerful tool in determining the prognosis of patients with acute lymphoblastic leukemia (ALL). Multiple methods to detect MRD exist, each with its own benefits and drawback. Multiparameter flow cytometry and quantitative polymerase chain reaction are the most commonly used methods of MRD detection in clinical practice. **Objective:** to evaluate the impact of minimal residual disease detection by Next Generation Flow Cytometry on Outcome of Egyptian Patients with Acute Lymphoblastic Leukemia. **Patients &Methods:** The study conducted on 93 patients with recently diagnosed acute lymphoblastic leukemia. MRD detection was evaluated during follow up of patient (at End of induction EOI and End of consolidation EOC by next generation flow cytometry. **Results:** Out of 93 patients, 28 (30%) had positive MRD at EOI. Age, BCR-ABL, risk assessment, and relapse had a substantial impact on MRD at EOI (P <0.005). Fourteen patients (17.9%) at EOC were MRD positive; age, hemoglobin, blast count at diagnosis, BCR-ABL, risk stratification, relapse and overall survival showed significant association. **Conclusion:** Positive MRD was a major risk factor for predicting poor survival and relapse at both EOI and EOC by cox regression analysis.

  Keywords: Acute lymphoblastic leukemia- Minimal residual disease- Outcome- Flow cytometry

Introduction

The malignant transformation and multiplication of lymphoid progenitor cells in the bone marrow, blood, and extramedullary locations is known as acute lymphoblastic leukemia (ALL). Even though 80% of ALL cases are in youngsters, it is a terrible disease when it affects adults. According to estimates, there are 1.6 cases of ALL for every 100,000 people in the United States (Chen & Wood 2017).

Owing to the application of risk-adapted therapy and improved supportive care, the five-year survival rate for pediatrics with ALL has considerably increased from 57 to 92% (Della et al., 2019). However, relapses still develop in 20%of children with ALL and are associated with a poor outcome (Tzoneva et al., 2013). A regular evaluation of treatment response gives a clue as to how responsive leukemic cells are to chemotherapy and how effective the treatment is all around. An essential duty in the clinical care of ALL patients, morphological assessment of bone marrow aspirates has historically been used to track treatment response (Bradstock et al., 1981).

Numerous studies have demonstrated that MRD could be considered the main prognostic predictor in children and adults with ALL. Monitoring disease burden in the context of stem cell transplantation (SCT), for early detection of impending relapse, and as a potential end point in studies are other uses for MRD detection in addition to assessing initial treatment response and defining MRD-based risk categories. MRD is employed in current treatment procedures to direct therapeutic decisions (Brüggemann& Kotrova 2017).

The sensitivity of the MRD analysis is ranging from 0.1 to 0.001% (10−1 to 10−5) for MFC and 0.001 to 0.0001% (10−5 to 10−6) for molecular techniques. The NGS technique could be considered a precise and sensitive approach of MRD evaluation, feasible to be used in the majority of cases with ALL. On the other hand, it has been demonstrated to be associated with high cost which is a main drawback for its broad applicability. The proper identification of immunoglobulin and T cell receptor gene rearrangements by quantitative real-time polymerase chain reaction (RT-PCR) has been considered as the best standard of care in terms of ALL management (Pawinska

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et al., 2022). However, the majority of current treatment strategies use an integral approach, applying both MFC and PCR techniques to evaluate MRD in ALL (Riva et al., 2021).

Wide applicability in ALL, together with availability in many laboratories, and rapid results in 24 h make MFC a broadly utilized modality in the context of MRD assessment. Therefore, MFC MRD monitoring is a well-established standard of care for early response evaluation in ALL in the USA and Europe (Schrappe et al., 2018). Owing to its relatively low cost and being a well-standardized technique, the MFC MRD strategy is also recommended in centers with limited resources (Pedrosa et al.,2020). In this study we try to evaluate the impact of minimal residual disease detection by next generation flow cytometry on patient outcome with acute lymphoblastic leukemia in Oncology Centre Mansoura University.

Materials and Methods

Patients

Between June 2020 and June 2021, Ninety -three consecutively recruited patient, aged 1–52 years, with recently diagnosed ALL who were managed in the oncology center, Mansoura University, Mansoura, Egypt.

The Risk stratification classification of patients was based on (age, WBCs, immunophenotyping, and CNS-infiltration). Our patients were favorable in 56 patients and unfavorable in 37 patients for children and adults, respectively. Patients with Acute myeloid Leukemia, Acute leukemias of ambiguous lineage, Other lymphoid or hematological malignancy and Relapsed cases of ALL were excluded in the study. All participants gave informed consent to their participation in this study. The current study was conducted accordance with the code of ethics of the world medical association (declaration of Helsinki) for studies comprising humans.

Diagnosis of ALL was done according to 2016 WHO criteria (Arber et al., 2016)

All patients were subjected to cytomorphology, immunophenotyping of blast cells, cytogenetic and molecular genetics. Flow cytometry assessment was performed at diagnosis. Peripheral blood or Bone Marrow (BM) samples were processed by utilizing bulk -lyse and stain method as defined (Tembhare et al.,2018). Samples were stained with 8-color acute leukemia panel this panel include 7 tube each contain 8 different markers by different color. Analysis was conducted on Navios EX Flow Cytometer from Beckman coulter. This instrument set up was checked every day by utilizing QC check beads (flow check profluorescence beads) purchased from Beckman coulter. Standard acquisition protocol was utilized for each experiment. Acquisition of 100,000 events was done at diagnosis. leukemia associated immunophenotyping (LAIP) was determined, at diagnosis, utilized afterwards for MRD measurements. monoclonal antibodies from (BD).

MFC- MRD monitoring

MFC-MRD was performed in BM samples at both at end of induction (EOI) and End of consolidation therapy (EOC) in bone marrow samples

A panel of monoclonal antibodies for MRD IN B-ALL CD 81FITC, CD 58PE, CD34 PE -CY5.5, CD 19 PE-CY7, CD 10 APC, CD 20 APC-H7, CD38 PB, CD45 KO.

In T-ALL CD 7 FITC, CD34 PE, CD3 PE-CY5.5, CD 4 PE-CY7, CD 1a APC, CD 8 APC-H7, CD5 PB, CD 16 FITC, CD56 PB, cCD 3 PE -CY7, CD2 APC-H7, CD99 PE, CD45 KO.

Staining technique was done according to manufacturing guidelines (BD).

Gating Strategy

Acquisition of1,000,000 events at MRD detection at both EOI&EOC

A-B-ALL

Initial gating was done using forward scatter area on X-axis versus forward scatter height on Y-axis to include only singlet cells, then another gate was done using CD45 versus forward scatter (SSC) to exclude debris and dead cells. A dot plot of CD19 versus SSC is gated on the above "viable" gate. A region is drawn around the CD19 population and labeled "CD19+ cells."

Normal mature B-cells that express CD10-/D20+ cells, plasma cells that express CD38 bright/CD10-, and normal early hematogones that express CD34 and bright CD10 are excluded. However, presence of clustered events expressed CD10, CD58, CD34, and dim (CD38, CD81) and dim CD45 and negative CD20, these cells considered as residual leukemic cells. These residual leukemic cells are confirmed by LAIP at diagnosis for each case.

Calculate limit of detection by dividing 20 per CD45 viable gate, a MRD positivity was identified based on an abnormal clustered event more than limit of detection (Figure 4).

B-T-ALL

Doublet exclusion result in singlet gating were followed by exclusion of debris, erythrocyte and platelet clumps on FSC/SSC plot (i.e., viable cell gating). Mononuclear cells were after that gated on SSC/CD7 scatter to exclude granulocytes, B-lymphocytes and monocytes. Mature T cells express a high level of surface CD3 with normal expression of CD4:CD8 ratio. While residual leukemic T cells frequently lack the surface expression of CD3 but express a cytoplasmic CD3. They may also exhibit decreased antigen expression or complete absence of markers like CD5, CD2, and dual negative or dual positivity of CD4 & CD8.Immature T cells markers as (CD34, CD1a, CD99) were determined according to LAIP at time of diagnosis. In order to accomplish lineage specificity, cytoplasmic CD3 is combined with surface CD3, and to rule out the typically subset of NK cells that could express cytoplasmic CD3, CD56 and CD16.

Statistical analysis

The baseline characteristics of both groups were compared by utilizing the Chi2 test. The survival analysis

was carried out through Kaplan-Meier method and the risk was measured with Cox regression. The Overall Survival (OS) was defined as the period of time from diagnosis to death and the Disease-free state (DFS) as the period of time from CR to relapse. Statistical analysis was performed by utilizing SPSS version 25.0. P<0.05 was considered as statistically significant difference.

Results

Patients' Characteristics

Ninety-three patients with ALL (37 female, and 56 males) were finally analyzed. The median age at the time of diagnosis was 10 years (range 1–52). The majority of the patients 74of 93 (79.6%) had (B-Cell Precursor) BCP-ALL, whereas 19 had T-ALL (20.4%). Only four patients had CNS infiltration, Risk stratification was favorable for 60.2% of the patients. All study patients underwent immunophenotyping at diagnosis, thus further MFC and MRD monitoring was feasible.

MRD was available at EOI in (93) patients and EOC in (78) patient

As regards MRD at EOI, MRD positive cases were significantly older than MRD negative cases. t (9;22) was significantly more frequent in MRD positive cases in comparison with MRD negative cases. Unfavorable risk stratification and relapse were significantly more frequent in MRD positive cases compared to MRD negative cases. While at EOC, MRD positive cases were significantly older than MRD negative cases. There was significant reduction of Hb and significant elevation of blast count in MRD positive cases compared to MRD negative cases. t (9;22) was significantly more frequent in MRD positive cases compared to MRD negative cases. Unfavorable risk stratification, relapse was significantly more frequent in MRD positive cases compared to MRD negative cases (Table 1).

MRD and Outcomes
A-According to MRD at EOI

The average follow-up was 22 months. All 93 patients attained (morphological remission blast<5%) after Induction chemotherapy. Patients who had negative MRD at EOI had significantly better 2-year OS than patients who were positive for MRD. OS estimated 92.3% in the MRD-negative group at 22 months against 43.9% in the MRD-positive group (P 0.002) (Figure1a). Additionally, they had a considerably decreased probability of relapse, with DFS estimating 97.8% in the MRD negative group at 20-month interval and 58.3% in the MRD positive group at the same interval. (P <0.001) (Figure 1b).

B-According to MRD at EOC

As regards EOC, cases with no residual disease cells detected at EOC was associated with a significant increase in 2-year OS than MRD positive patients 90.4% at 22months in MRD negative group versus 28.1% in MRD positive group (P 0.001) (Figure 2a).

On the other hand, DFS estimated 95.7% at 20 months interval in MRD negative group, also DFS estimated 49.2% at 20 months interval in MRD positive group with significant difference between 2 groups (P <0.001) (Figure 2b).

Cox regression analysis for predicting poor survival and relapse

Age, gender, laboratory data, type, leukaemia subtype, risk stratification, and MRD at both EOI and EOC were included as factors in a COX regression analysis to predict poor survival and relapse.

Regarding OS, the univariate analysis shows that WBCS count and positive EOI and EOC MRD were significant risk factors for OS. While, Positive EOI and EOC MRD were the only real risk factors for poor survival in the multivariate analysis. On the other side elevated Hb was a significant protective factor in univariate analysis, whereas older patients, unfavorable risk stratification,
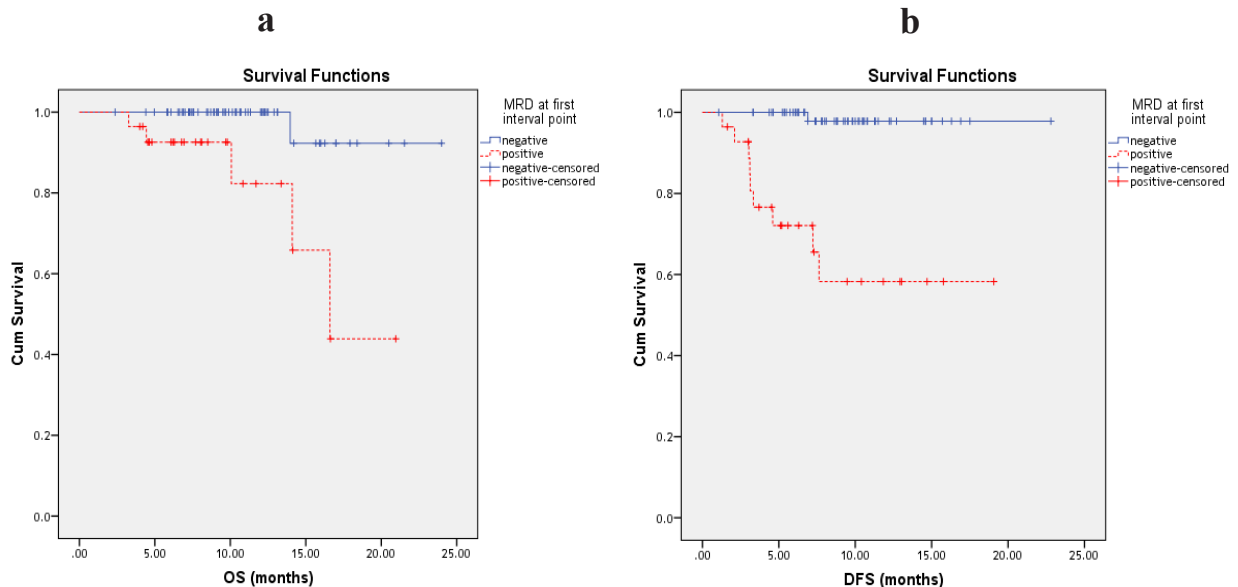
a **b**

Figure 1. a, Survival profile at EOI; b, DFS profile in ALL patients at EOI

Table 1. Comparison of Clinicopathological Characteristics and Laboratory Parameters as Regard MRD at End of Induction and End of Consolidation

		EOI			EOC		
		MRD negative (N=65)	MRD positive (N=28)	P-value	MRD negative (N=64)	MRD positive (N=14)	P-value
Age*	Median (Min-Max)	8.0 (1-50)	17.5 (2-52)	0.004	10.0 (1-50)	20.0(3-48)	0.005
Age group	<18	56 (86.2%)	16 (57.1%)	0.002	53(82.8%)	7(50.0%)	0.008
	>18	9 (13.8%)	12 (42.9%)		11(17.2%)	7(50.0%)	
Gender	Male, (N (%))	37 (56.9%)	19 (67.9%)	0.323	38(59.4%)	8(57.1%)	0.878
	Female, (N (%))	28 (43.1%)	9 (32.1%)		26(40.6%)	6(42.9%)	
Diagnosis	B-ALL	52 (80.0%)	22 (78.6%)	0.875	48(75.0%)	12(85.7%)	0.501
	T-ALL	13 (20.0%)	6 (21.4%)		16(25.0%)	2(14.3%)	
WBCS*	Median (Min-Max)	22.0 (1.3-284.0)	37.0 (1.8-436.0)	0.209	20.2(1.3-436.0)	47.5(2.8-434.0)	0.065
Hb**	Mean ± SD	9.0 ± 2.23	8.38 ± 1.97	0.192	9.06 ± 2.21	7.50 ± 1.94	0.017
PLT*	Median (Min-Max)	37.0 (4.0-453.0)	22.0 (6.5-165.0)	0.088	37.5 (4.0-453.0)	21.0 (8.0-165.0)	0.129
Blast %*	Median (Min-Max)	90.0 (22.0-97.0)	95.0 (37.0-98.0)	0.094	90.0 (22.0-97.0)	95.0 (85.0-98.0)	0.007
t (9;22)	N of positive (%)	1 (2.6%)	4 (25.0%)	0.023	2 (5.4%)	3 (33.3%)	0.044
t (12;21)	N of positive (%)	6 (15.8%)	0 (0.0%)	0.163	5 (13.5%)	0 (0.0%)	0.566
7q34	N of positive (%)	3 (30.0%)	3 (75.0%)	0.245	4 (33.3%)	2 (100.0%)	0.165
Risk stratification	Favorable	56 (86.2%)	0 (0.0%)	<0.001	46 (71.9%)	0 (0.0%)	<0.001
	Unfavorable	9 (13.8%)	28 (100.0%)		18 (28.1%)	14 (100.0%)	
Relapse	No relapse, (N (%))	64 (98.5%)	19 (67.9%)	<0.001	62 (96.9%)	8 (57.1%)	<0.001
	Relapse, (N (%))	1 (1.5%)	9 (32.1%)		2 (3.1%)	6 (42.9%)	
Outcome	Alive, (N (%))	64 (98.5%)	23 (82.1%)	0.009	62 (96.9%)	10 (71.4%)	0.008
	Dead, (N (%))	1 (1.5%)	5 (17.9%)		2 (3.1%)	4 (28.6%)	

and positive MRD at both EOI &EOC were significant risk factors for relapse. In the multivariate analysis, only positive MRD at both EOI and EOC were significant risk factors, while higher Hb was a significant protective factor for relapse (table 2).

Performance characteristics of MRD quantity at both EOI and EOC time point for discrimination of relapsed from non-relapsed cases (Table 3)

By ROC curve analysis the best cutoff of MRD at EOI was 0.28%. The area under the curve (AUC) was 0.924 (p=<0.001) (Figure 3a), while at EOC was 0.91. The area under the curve (AUC) was 0.958 (p=0.003) ((Figure 3b).

Discussion

Monitoring of MRD after induction and consolidation of acute lymphoblastic leukemia is a standard of care in several recent treatment strategies, in children as well as in adults. The proper assessment of EOI-MRD has been considered as the main prognostic factor (Contreras et al.,2021). The detection of immunoglobulin and T cell
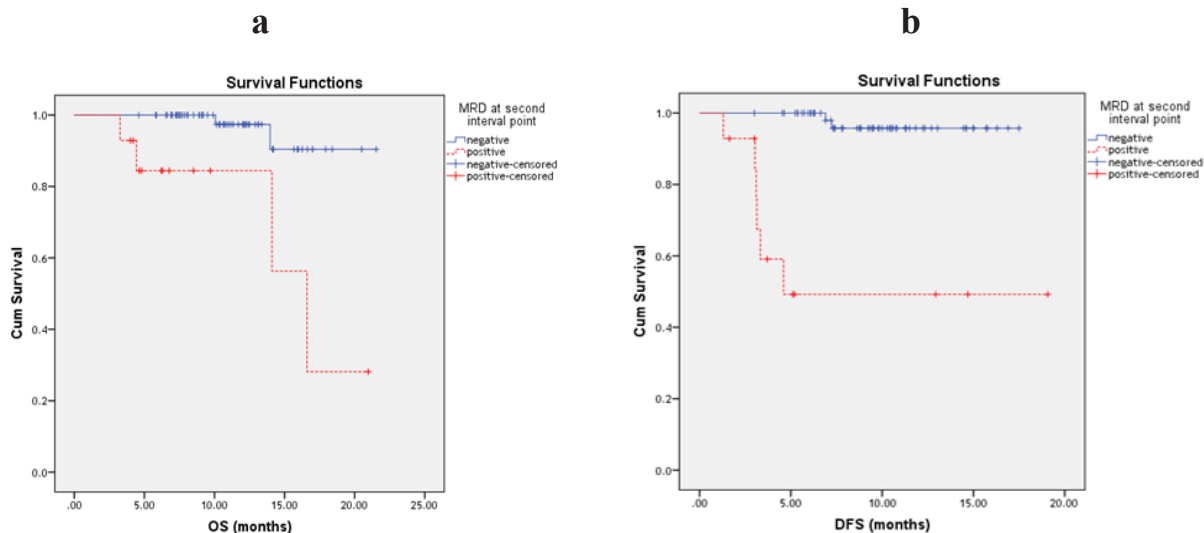
Figure 2. a, Survival profile at EOC; b, DFS profile at EOC

Table 2. Multivariate Analysis for Overall Survival and DFS

| | Cox regression analysis for prediction of shorter OS | | | | | | | | Cox regression analysis for prediction DFS | |
| | Univariate analysis | | | | Multivariate analysis | | | | Univariate analysis | |
	P	HR	95% CI		p	HR	95% CI		P	HR
Age	0.645	1.014	0.956	1.075					0.024	1.045
Gender	0.289	0.313	0.037	2.68					0.962	1.031
WBCS	0.01	1.008	1.002	1.015	0.103	1.006	0.999	1.012	0.159	1.004
Hb	0.954	0.984	0.708	1.443					0.003	0.63
PLT	0.405	0.987	0.958	1.018					0.064	0.962
BM blast	0.834	0.996	0.955	1.038					0.45	1.033
T ALL vs B ALL	0.06	4.763	0.939	24.175					0.907	1.912
Risk stratification (unfavorable vs favorable)	0.079	6.879	0.798	59.27					0.007	16.862
MRD at EOI	0.019	13.185	1.532	113.43	0.032	11.023	1.225	99.21	0.001	29.172
MRD at EOC	0.007	10.971	1.938	62.093	0.025	8.085	1.303	50.15	<0.001	26.016

a **b**
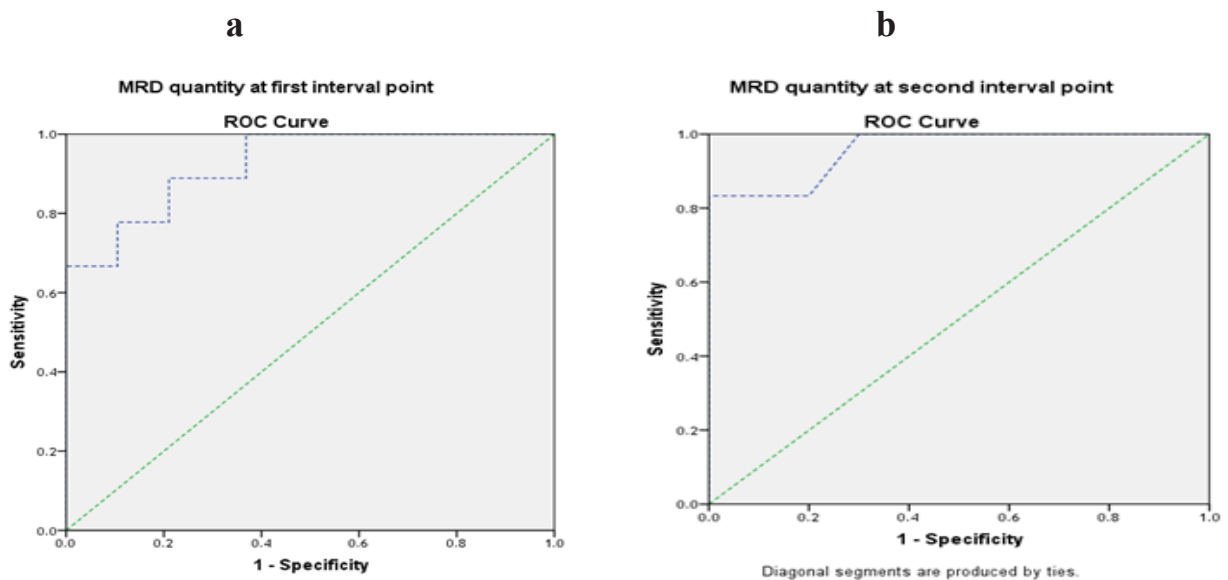
Figure 3. a, MRD quantity at EOI; b, MRD quantity at EOC

receptor gene rearrangements by quantitative real-time PCR (RT-PCR) is considered best approach for MRD assessment (Kruse et al.,2020). However, MFC method is more accessible and reveals acceptable performance (Burnusuzov et al.,2016).

Regardless of the method used, MFC, PCR, or NGS, the evaluation of MRD was considered as a sensitive modality in pediatric cases with ALL to recognize cases for whom HSCT is indicated in the first CR and those who could be managed with standard chemotherapy only (Schrappe et al.,2018). The accurate time points at which MRD is measured and the threshold used for treatment decisions differ between therapeutic strategies and the

MRD detection method (Pui et al.,2017).

We performed MRD at two time points during follow up of the patient with acute lymphoblastic leukemia. The first at end of induction of chemotherapy (EOI) and the second at end of consolidation (EOC) and this was supported by Schumich et al who reported that MRD at the (EOI) and (EOC) has been demonstrated to be a significant prognostic tool in several international studies (Schumich et al.,2019). Similarly, Stock et al noted that post induction and consolidation MRD were established as a main prognostic factor in ALL (Stock et al.,2015).

Contrarily, another major study demonstrated that the bone marrow measurement of the MRD by the MFC

Table 3. Performance Characteristics of MRD Quantity at Both EOI and EOC for Discrimination of Relapsed from Non-Relapsed Cases

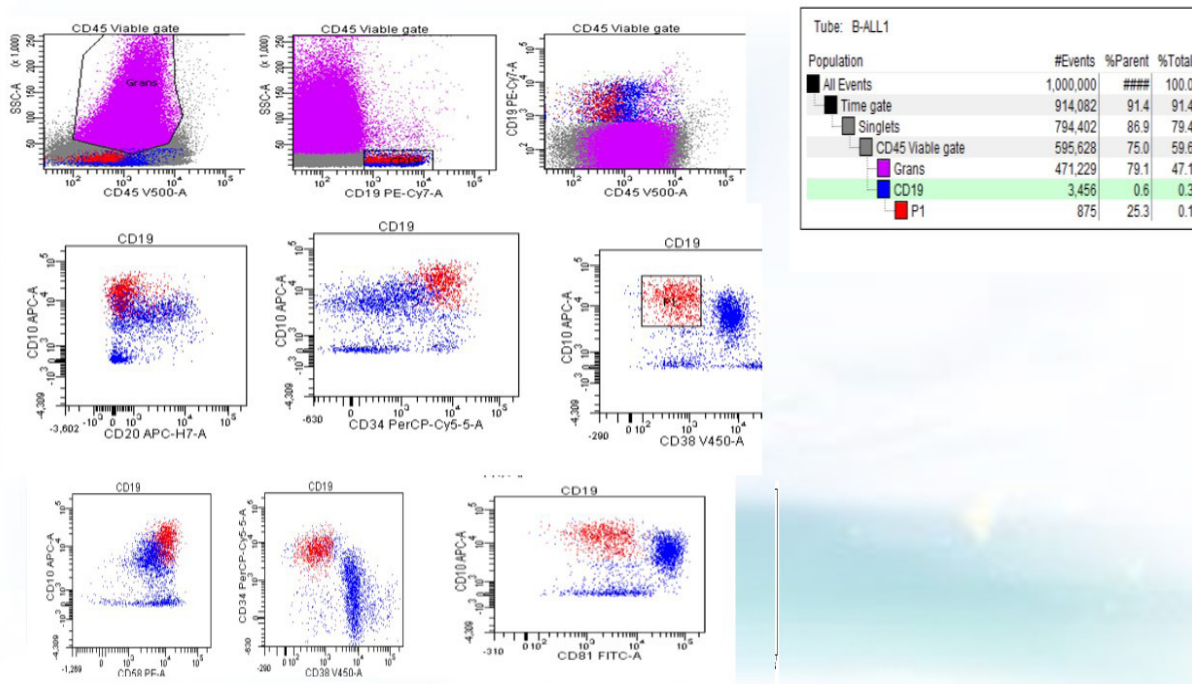|	AUC	SE	p	95% CI	Cut off	Sensitivity (%)	Specificity (%)
MRD quantity at EOI	0.924	0.051	<0.001	0.823-1.00	0.28	88.90%	78.90%
MRD quantity at EOC	0.958	0.049	0.003	0.863-1.00	0.91	83.30%	100.00%

Figure 4. Represents a Positive (MRD) B-ALL Case by Next Generation Flow-Cytometry: residual leukemic cells in red dots (express CD10,CD34,CD58 and dim CD38,CD81 and negative CD20)

on D15 also has a potent prognostic impact and may complement risk stratification (Basso et al.,2009). Sutton et al proclaimed that early MRD measurement at day 15 in childhood ALL can provide further data for identification of very early responders (<10-3) and a small subgroup of poor responders (>10-2). However, so far there is no consensus about utilization of this early MRD detection in therapeutic decision making (Sutton et al.,2009).

We sought to determine the effects of minimal residual disease detection using next-generation flow cytometry on the prognosis of Egyptian patients with acute lymphoblastic leukaemia. We also evaluated MRD at two time points and determined which of them was more sensitive to predict relapse.

In the current study, our results of (MRD) of ALL patients at EOI revealed that out of our 93 patients 64 (70%) had negative MRD and 14 (30%) had positive MRD. This is in line with the result from Maraj et alwho found that 63.3% of ALL patients achieved negative MRD after finishing induction chemotherapy (Meraj et al.,2020) Furthermore, a pediatric study by Children's Oncology Group reported that 28.6% end induction MRD positivity (Borowitz et al.,2003).

According to clinic-biological features of patients at diagnosis, our study revealed that MRD positive cases at EOI and EOC were significantly older. This agrees with Coustan et al report that residual disease was significantly more frequent in pediatrics 10 years of age or more (Coustan et al.,2000).

In the current study, MRD detection on completion of induction therapy and consolidation was not significantly related to gender, initial WBCs count or type of leukemia. and this in agree with Jovanovska et al who reported, that the presenting characteristics comprising sex, WBC count at diagnoses, neurological affection, immunophenotype

didn't vary in a significant manner between cases with negative and positive MRD status at the EOI therapy (Jovanovska et al.,2019). this may be related to the inclusion of both pediatric and adult groups with different favorable factors for each group.

Likewise, cytogenetic abnormalities characteristic of B-ALL mainly determine the diseases biology, affect prognosis, and guide therapy (Meraj et al.,2020). Cytogenetic test by Fluorescence In Situ Hybridization (FISH) was done for 54 of our patients t (9:22, t12:21,11q23). And we found that t9:22 was significantly associated with positive MRD at both EOI and EOC and this was confirmed by other published studies by van Dongen et al who showed that t (9:22) to be a bad predictor with a greater possibility of relapse and MRD positivity (van Dongen et al.,2015).

The prognostic value of minimal residual disease (MRD) at EOI and EOC was also evaluated in this study. The 2-year Disease free survival (DFS) for MRD negative patient at EOI were 97.8%versus 58.3% MRD positive patient (p value 0.001). So MRD negative group demonstrated a significant reduction in the possibility of relapse in comparison with MRD positive. Moreover, at EOC were 95.7% in MRD negative versus49.2% MRD positive patient (p value< 0.001) and this come in agreement with Silva et al who reported that there was a significant correlation between MRD positivity and relapse (Silva et al.,2020).

In univariate analysis, older patients, presence of unfavorable risk factors, and positive MRD at both EOI &EOC were significant risk factors for relapse, whereas increased Hb was significantly protective against relapse. In the multivariate analysis, the only independent risk factors for relapse were positive MRD at EOI and EOC, while higher Hb was a significant protective factor.

On the other hand, Overall Survival (OS) was also evaluated in our study and revealed that OS was significantly higher among MRD negative group at (EOI and EOC) In line with our finding, Liao et al who assessed MRD by next generation flow cytometry and PCR on 486 adult ALL patient and found a significant association between EOI MRD and patient outcome (Liao et al.,2022).

Age, WBC, Hb, Platelet, blast in the bone marrow at diagnosis, Leukaemia subtype, risk stratification and MRD positivity at both EOI and EOC were all factors that were evaluated in our study for the prediction of poor survival. We discovered that in a univariate analysis, a high WBCS count and a positive MRD at both EOI &EOC were significant risk factors for poor survival but in the multivariate analysis, only MRD at both EOI &EOC were significant risk factors for poor survival.

Our study confirmed that the importance of MFC MRD at EOI in the BM was the most powerful early predictor of relapse by ROC curve analysis. It plays an essential role to determine the most precise assessment time point featured by the highest sensitivity to predict relapse at EOI, EOC was demonstrated to be identically significant (AUROC EOI vs. AUROC EOC, 0.924 vs. 0. 958.And this agrees with Pawinska et al who - confirmed that MRD on day 33 end of induction in the BM as the most powerful early predictor of relapse (Pawinska-et al., 2022). Contrarily Conter et al who used MRD (0.1%) at end-consolidation for risk stratification. Of note, their study only included children and adolescents and they evaluated MRD by a different technique (PCR) (Conter et al., 2010).

Regarding the best cutoff values for diagnosing relapsed cases, 0.28% was the best cutoff for differentiating relapsed from non-relapsed cases at EOI and 0.91% at EOC. This is in agreement with Pawinska et al. who evaluated MRD at three time points (D15, D33, and D78) and discovered that the 0.1% MRD cut-off was the most discriminatory for entire cases at all- time points evaluated (Pawinska et al.,2022). In contrast, Borowitz et al suggested that 0.01% was a better cut-off at end of induction (Borowitz et al.,2015).

In conclusion the current study found that MRD negativity at EOI and EOC were significantly associated with favorable prognostic factors, treatment outcome and better survival. Furthermore, our results displayed that MRD at end of induction was more sensitive predictor for relapse than subsequent MRD done later at end of consolidation.

Author Contribution Statement

Reem Algamal, Nashwa Abousamra and Doaa Shahin: Conception, Interpretation and analysis of data preparation of the manuscript, revision, supervision. Reem Algamal, Rasha Elashary and Suzy AbdElmabood: assisted in samples collection and follow up of patients during the study.

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Ethical committee
Our study has been approved by IRB of corresponding university (Code Number: MD.20.05.325).

Disclosure
The authors have no conflicts of interest to declare.

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