FACTORS INFLUENCING CLEARANCE OF LEUKAEMIC CELLS ON DAY 28 BONE MARROW ASPIRATE IN PAEDIATRIC B- ALL PATIENTS

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ABSTRACT

BACKGROUND
In India, leukaemia continues to be the largest contributor to cancer-related mortality in children. The potential applications of minimal residual disease studies in the clinical management of acute leukaemia include early identification of patients at a higher risk of relapse.

The aim of the study was to determine-
1. The extent of clearance of leukaemic cells as assessed by Peripheral blood, Bone marrow aspirate on day 28 and Flow cytometry in paediatric B- ALL patients.
2. Its association with standard prognostic variables.

MATERIALS AND METHODS
Immunophenotyping with flow cytometry (4 colour) was used along with peripheral smear, bone marrow, clinical and laboratory details in a prospective cohort study among paediatric B-cell ALL patients in a tertiary level referral centre from December 2014 - June 2016. Statistical analysis was done using SPSS 18.

RESULTS
Analysis of 35 paediatric B- ALL cases showed that those with central nervous system involvement at the time of diagnosis had more chance of minimal residual disease positivity after induction chemotherapy (p=0.001). The patients who showed blasts in their day 7 peripheral blood also had MRD (p=0.001). This study also showed that CD34 down modulation showed a positive correlation with presence of MRD (p=0.048).

CONCLUSION
The patients assigned to standard risk category by conventional prognostic factors will benefit by the detection of MRD by flow cytometry at the end of induction chemotherapy. MRD detection using flow cytometer (4 color) will provide a basis for future clinical decision making in the management of ALL cases.

KEYWORDS
Lymphoblastic Leukaemia; Minimal Residual Disease; Flow Cytometry; Risk Stratification.


BACKGROUND
Acute leukaemia is the most common paediatric haematological malignancy worldwide. It arises from malignant transformation of haematopoietic stem cells.1 In India, leukaemia is the largest contributor to cancer-related mortality in children.2

About 80% of all leukaemia reported in children is acute lymphoblastic leukaemia.3 ALL peaks in incidence in the age group of 1 to 5 years.4 Males are affected more frequently than females.5 In ALL, relapse represents the main cause for treatment failure. Early response to therapy is a primary prognostic factor in ALL.6

Minimal Residual Disease (MRD) is defined as persistence of resistant malignant cells in the bone marrow and/or peripheral blood in patients in continuous clinical remission. The potential applications of MRD studies include early identification of patients at a higher risk of relapse.

Although, microscopic examination of the bone marrow has traditionally been used to identify remission, it is difficult to detect levels of leukaemic infiltration below 5%. Thus, patients maybe in remission by traditional criteria, but still have a large tumour burden, i.e. these patients are still harbouring 10^10 cells in the marrow at this stage.7

The cut-off level commonly used to define MRD positivity is 0.01% of bone marrow mononuclear cells, because this is typically the limit of detection for routine FCM assays.8 In patients with ALL, treatment response is increasingly evaluated with MRD assays, for which FCM can be used.9 Multiparameter flow cytometry is used to
distinguish leukaemic cells from normal. The patients with an MRD level of 0.01% or higher in bone marrow by flow cytometry at any treatment interval had a significantly higher risk of relapse. Currently, the most widely used methods for the detection of MRD in the bone marrow and/or peripheral blood are PCR and FCM. MRD is the most important prognostic indicator for relapse.

MRD more than or equal to 0.01% on day 29 by flow cytometry was the strongest prognostic indicator in studies of the Children's Oncology Group. Measurements during remission induction therapy provide an early identification of good responders and of very poor responders. The immunomodulation of the different antigens expressed by the leukaemic blasts should be taken into consideration.

Aims and Objectives
1. To determine the extent of clearance of leukaemic cells as assessed by peripheral blood, bone marrow aspirate on day 28 and flow cytometry in paediatric B- ALL patients.
2. To determine its association with variables like age, gender, WBC count, lymph node enlargement, hepatosplenomegaly, CNS involvement, testicular enlargement and FAB morphology.

MATERIALS AND METHODS
It was a prospective cohort study of children with B- ALL during December 2014 - June 2016 at Department of Pathology/Paediatrics, Government Medical College, Kozhikode.

a) Inclusion criteria - all children diagnosed as B-ALL in Leukaemia Unit, Institute of Maternal and Child Health, Kozhikode, based on bone marrow aspirate immunophenotyping by FCM in Department of Pathology were included.
b) Exclusion criteria - Children who were being treated in other centers were excluded.

Methodology - The patients were included in the study after getting informed consent from the parent. The samples for haematological investigations were received in the Department of Pathology as a part of routine investigation during the period of December 2014 to June 2016. Clinical data, peripheral blood film morphology, bone marrow aspirate morphology, flow cytometry data and treatment details were recorded.

The peripheral blood and bone marrow samples on day 28 of induction therapy were collected. The peripheral blood film and the bone marrow aspirate were examined under light microscopy. FCM was done on bone marrow aspirate. Immunophenotyping at the time of diagnosis was done using markers like CD45, CD19, CD34, CD10, CD117, CD13, CD33, HLA-DR, TdT, cCD3, CD5, CD77, cCD79a and MPO. MRD detection in bone marrow on day 28 was done using 7 tubes using the markers; CD45, CD19, CD10, CD20, CD13, CD34, CD38, CD58 and CD123. All cases were done in the 4 colour flow cytometer with the panel and fluorochromes as shown in Figure 1.

The cases were also analysed for presence of blasts in CSF by cytocentrifugation.

Cytogenetic study was also done in the Department of Paediatrics as a part of the routine investigations of patients with acute leukaemia for their risk stratification.

Modified BFM-95 protocol for high-risk and low-risk groups was used. The risk was taken as standard risk when-
- Age was between 1 year and 10 years.
- Total count was <50,000.
- Morphological type L1 or L2.
- No CNS involvement.

High risk included those patients with-
- Age of <1 and >10 years.
- WBC count of >50,000.
- L3 morphology.
- CNS involvement.
- Blasts on day 7 of starting treatment.

Data Interpretation - In light microscopy, <5% blasts was considered as leukaemia clearance in bone marrow. In FCM, CD19 positive cells with dimCD45, dimCD38, cells with over expressed CD58, CD123, cells with alteration in expression of CD10 or CD20 (when compared to earlier expression at the time of diagnosis) and cells with aberrant expression of myeloid marker CD13 were considered as abnormal population.

The possible population of MRD events was identified in the patient tubes by comparing with haematogone patterns in ITP control marrow samples. 10 cases of ITP marrow samples were collected from age-matched cases. This was to standardise the haematogone population. These marrow samples were received in the Department of Pathology as a part of routine investigations of ITP. The maximum of events identified in the area marked for blasts in controls (ITP) and cases were compared. Percentage of those events in the total gated events was then calculated.

The data regarding variables like age, gender, white blood cell count, lymph node enlargement, hepatosplenomegaly, central nervous system involvement, testicular enlargement and FAB morphology were entered in a structured pro forma and their association with clearance of leukaemic cells were assessed.

Statistical Analysis - The data was analysed using SPSS 18.0 statistical software. The prevalence of minimal residual disease in B-ALL was expressed as percentage. The association between clearance of leukaemic cells and various variables was assessed using Chi-square test and p values <0.05 were considered significant.

RESULTS
The total number of patients were 35, out of which, 22 were males. The mean age of the patients was 5.14 years (3 months to 12 years). The mean WBC count at diagnosis was 67,200. 13 (37.14%) had count >50,000. The minimum count observed in our study was 1,880/cmm and maximum count was 4,30,000/cmm.
22 (62.86%) patients showed significant lymphadenopathy in cervical and axillary regions. 27 (77.14%) patients presented with hepatosplenomegaly. 5 (14.3%) patients had CNS involvement. 8 (22.9%) patients showed blasts with FAB L1 morphology and 27 (77.1%) showed L2 morphology. Out of 35 patients, none had testicular enlargement. Aberrant marker expression was present in the patients as shown in Figure 3. Only 1 out of 35 patients had positive translocation, i.e. t (9, 22).

13 patients were standard risk and 22 were high risk. Five (14.3%) out of 35 patients had blasts in peripheral blood on day 7 of induction chemotherapy. All 35 patients had no blasts in peripheral blood on day 15 and day 28 of induction chemotherapy. All had <5% blasts in their day 28 bone marrow aspirate.

It was shown that 85.7% children had CD34 positivity and 94.3% children had blasts with CD10 positivity at the time of diagnosis. All patients showed CD19 positivity at diagnosis. 12 (34.3%) patients had MRD (>0.01%) at the end of induction phase.

Chi-square test showed no significant correlation between various variables and presence of MRD as shown in Table 1.

It was shown that 23 (76.7%) of the 30 patients who did not have day 7 peripheral blood blasts had no MRD at the end of induction phase. 7 patients who did not have blasts in day 7 peripheral blood showed positive MRD. All the 5 patients who showed blasts in their peripheral blood on day 7 of induction phase had detectable MRD with a p value of 0.001 at the end of induction phase.

Similarly, all the 5 patients who showed CNS involvement at the time of diagnosis had detectable MRD at the end of induction phase (p=0.001).

Comparison of one of the cases (with positive MRD) and controls are shown in Figure 2 and 3. Two patients who had positive MRD showed CD34 down modulation at the end of induction therapy as shown in Figure 4 (p=0.048). There was no alteration in expression of CD10 and CD19.

### Table 1. Correlation of Variables with Presence of MRD

<table>
<thead>
<tr>
<th>Variable</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.906</td>
</tr>
<tr>
<td>Gender</td>
<td>0.761</td>
</tr>
<tr>
<td>WBC count</td>
<td>0.566</td>
</tr>
<tr>
<td>LN enlargement</td>
<td>0.566</td>
</tr>
<tr>
<td>Hepatosplenomegaly</td>
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<tr>
<td>Aberrant marker</td>
<td>0.832</td>
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<tr>
<td>Cytogenetics</td>
<td>0.453</td>
</tr>
<tr>
<td>FAB morphology</td>
<td>0.881</td>
</tr>
<tr>
<td>Risk category (high and low risk)</td>
<td>0.354</td>
</tr>
</tbody>
</table>

### DISCUSSION

The age group in our study was very much comparable to the median age of 6 years in Karachi study and another study conducted by Rana et al in which mean age was 5.4 years. In many studies, ALL peaks in incidence in the age group of 1 to 5 years.

In a study conducted by Yasmeen et
al in Karachi, males were 64% and females 36%.\textsuperscript{17} This study was also in concordance with our study.

The study by Azma et al showed only 11% children with WBC count >50,000/cmm. Karachi study showed 34% patients with >50,000 initial WBC count. This study goes hand in hand with our study. The Malaysian study showed 76% of patients with lymph node enlargement at presentation. The study by Yasmeen et al also showed 75% patients with lymphadenopathy at the time of initial diagnosis.\textsuperscript{15} In the study conducted by Azma et al showed hepatosplenomegaly in 85% patients and study by Rana et al showed 67% patients with hepatomegaly, which is comparable to this study.\textsuperscript{18}

In a study conducted in Sweden by Ranta et al, there was 17.7% CNS involvement.\textsuperscript{19} Another study by Rana et al, 5% children showed CNS involvement. A study by Larson et al demonstrated 37% patients having blasts of L1 morphology and 46% with L2 morphology.\textsuperscript{20} In the Malaysian study by Azma et al, 56.7% patients had aberrant myeloid antigen expression at diagnosis and 43.3% had no aberrant marker expression, which is comparable to our study.

In a study of 434 children by Fletcher et al, only 15 children had 9:22 translocation, i.e. 3.4%.\textsuperscript{21} According to Pui and Campana, the prevalence of this translocation was 4-6% in children with B-cell ALL.\textsuperscript{22}

In a study conducted by Coustan Smith et al, 57.8% were standard risk and 42.2% were high risk.\textsuperscript{23} In a study conducted in Tokyo, 15% patients showed blasts in their day 7 peripheral blood, which was comparable to our study.\textsuperscript{24} A study of 546 patients with childhood ALL showed 57 (14%) children with blasts in their day 15 bone marrow.\textsuperscript{25} All children were in remission on day 28 of their induction chemotherapy by morphological criteria in this study. In the study by Rana et al, 74% patients went into complete remission (<5% blast cells in bone marrow), 5% into partial remission (5-25% blast cells in bone marrow) and 3% was not in remission (>25% blast cells in the bone marrow).\textsuperscript{16}

In this study, CD19, CD10 and CD34 were the markers used commonly at the time of diagnosis and at the time of MRD detection. CD19 was positive in all cases at the time of initial diagnosis. In a study by Chen et al, 98.9% patients had blasts with CD19 positivity at the time of diagnosis.\textsuperscript{26} CD10 antigen was detected in blast cells from 384 of 408 patients (94%) with B-lineage ALL in a study conducted by Pui et al\textsuperscript{27} and it was associated with favourable presenting features in that study. CD34 antigen was detected on blast cells in 235 (70%) of 335 cases of newly-diagnosed childhood acute lymphoblastic leukaemia in another study conducted by Pui et al.\textsuperscript{28}

12 patients had MRD >0.01% at the end of induction phase with a mean value of 0.046% (maximum of 0.6% and minimum of 0%) in our study. In a study by Coustan Smith et al, 54% children had MRD >0.01% at end of induction therapy. Bulgarian study showed 75% children with >0.01% MRD on day 33.
CONCLUSION
The patients assigned to standard risk category by conventional prognostic factors will benefit by the detection of MRD by FCM at the end of induction chemotherapy by using more advanced 8 or 10 colour flow cytometer. But, in a resource poor setting, 4 colour flow cytometry technique described by us would be beneficial. For better evaluation of association of standard prognostic factors and MRD, a larger cohort of ALL cases is required.

REFERENCES

