Relevance of flow cytometric categorization and end-of-induction measurable residual disease assessment in pediatric and adult T-lymphoblastic leukemia patients

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Background
T-lymphoblastic leukemia (T-ALL) patients expressing myeloid/stem cell antigens are classified as early T-cell precursor lymphoblastic leukemia (ETP-ALL) or near-ETP-ALL.

Methods
Clinico-laboratory profiles, flow cytometric end-of-induction measurable residual disease (EOI-MRD), and survival of treatment naïve T-ALL patients were analyzed according to their immunophenotypic subtypes.

Results
Among 81 consecutive T-ALL patients diagnosed, 21% (N=17) were ETP-ALL and 19% (N=15) were near-ETP-ALL. EOI-MRD was detectable in 39% of the 59 samples tested (31.6% of pediatric samples and 52.4% of adult samples). The frequency of EOI-MRD positivity was significantly higher among ETP-ALL (75%, P=0.001) and near-ETP-ALL (71%, P=0.009) patients compared to that in conventional-T-ALL (con-T-ALL) patients (22.5%). CD8 (P=0.046) and CD38 (P=0.046) expressions were significantly upregulated in the EOI blasts of con-T-ALL and ETP-ALL samples, respectively. The 2-year rates of overall (OS), relapse-free (RFS), and event-free survival (EFS) among the T-ALL patients (pediatric vs. adult) was 79.5% vs. 39.8% (P<0.001), 84.3% vs. 60.4% (P=0.026), and 80.3% vs. 38% (P<0.001), respectively. Univariate analysis revealed that 2-year EFS and RFS of pediatric T-ALL patients was independent of T-ALL subtype and was influenced only by EOI-MRD status. However, 2-year OS, RFS, and EFS among adult T-ALL patients were EOI-MRD independent and influenced only by the near-ETP-ALL phenotype.

Conclusion
Two-year survival among pediatric and adult T-ALL patients is attributed to EOI-MRD status and near-ETP-ALL phenotype, respectively.

Key Words
Measurable residual disease, Flow cytometry, T-lineage acute lymphoblastic leukemia, ETP-ALL, Near-ETP-ALL

INTRODUCTION

T-lymphoblastic leukemia (T-ALL) comprises 15% of pediatric and 25% of adult acute lymphoblastic leukemia patients [1]. First described by Coustan-Smith et al. [2] in 2009, ‘early T-cell precursor lymphoblastic leukemia’ (ETP-ALL) is a subtype of T-ALL in which T-lymphoblasts express myeloid/stem cell-associated antigens in the absence of CD1a, CD5, and CD8 expression. ‘Near-ETP-ALL’ is a T-ALL subtype recognized by the World Health Organization (WHO) in 2017. In this subtype, T-lymphoblasts meet all immunophenotype criteria for ETP-ALL, except for having significant CD5 expression [1-3]. Clinical and laboratory characteristics of pediatric and adult ETP-ALL patients have been documented. However, data of near-ETP-ALL patients...
are limited [4, 5]. Also, the role of other lineage-specific and non-lineage-specific antigens that could distinguish between T-ALL subtypes is unknown.

Flow cytometric measurable residual disease (FCM-MRD) assessment is important for the risk-adapted management of B-lymphoblastic leukemia (B-ALL) patients. However, FCM-MRD-based treatment decisions are not yet part of the management protocols for T-ALL patients. This reflects the limited availability of literature on FCM-MRD in T-ALL. Most of the available publications have included both ETP-ALL and near-ETP-ALL as a common category for data analysis [4, 6-9].

To the best of our knowledge, data comparing age group specific clinico-laboratory profiles across the immunophenotypic subcategories of T-ALL patients are still lacking. Presently, we share our experience regarding clinico-laboratory profiles, end-of-induction (EOI) FCM-MRD, and 2-year survival outcomes of pediatric and adult T-ALL patients immunophenotypically subclassified according to the WHO 2017 guidelines.

**MATERIALS AND METHODS**

This retrospective study was approved by our Institute’s ethics committee. All treatment naïve T-ALL patients diagnosed between December 2017 to March 2020 were included. T-ALL was diagnosed by morphologic evaluation of peripheral blood (PB) and bone marrow (BM) aspiration smears, followed by a 10-color FCM analysis (Supplementary Table 1). Hyperleukocytosis was defined as ≥100×10⁹/L leukocytes in PB [7]. Pediatric (age ≤18 yr) and adult patients were treated with the Indian Collaborative Childhood Leukemia group (high risk-arm) and Berlin-Frankfurt-Muenster (BFM) 95 protocols, respectively [10]. Treatment protocols were not influenced by T-ALL immunophenotype subtype or end-of-induction measurable residual disease (EOI-MRD) status. During induction, an absolute PB blast count ≥1,000 cells/μL on day 8 of treatment was considered ‘day 8 blasts not cleared’ (D8BNC) status [11, 12].

**Diagnostic flow cytometry**

BM samples were processed using our previously described ‘lyse-stain-wash’ protocol [13]. A minimum of 100,000 events were acquired per tube using a Beckman Coulter Navios EX flow cytometer. Generated list-mode data (LMD) files were analyzed with Kaluza (Version 2.0) software (Beckman Coulter) using our in-house developed analysis templates. The antigen expression profile was reported according to the Associazione Italiana Ematologia Oncologia Pediatria-BFM (AIEOP-BFM) 2016 recommendations [14]. The expression intensity of each antigen was assessed by the geometric mean (GM) of expression determined by the Kaluza software.

Diagnoses of ETP-ALL and near-ETP-ALL used published criteria [1, 3, 4, 6, 15]. Patients not fulfilling the criteria for ETP-ALL or near-ETP-ALL were designated ‘conventional’ T-ALL (con-T-ALL). The intensity of CD5 expression on blasts was determined as the ratio between CD5-GM of T-lymphocytes within the sample to the CD5-GM of blasts (T-CD5: Bl-CD5 ratio) [15]. Our algorithm for classifying T-ALL patients into immunophenotypic subcategories is described in Supplementary Fig. 1A.

**Flow cytometric MRD assessment**

EOI-MRD was assessed in first-pull bone marrow aspiration (BMA) samples. The BMA samples were bulk-lysed with in-house prepared ammonium chloride-based lysis reagent and stained with an 11-antigen, 10-color cocktail (Supplementary Table 1). The processed samples were immediately fixed with 0.5% paraformaldehyde and acquired until the tube ran dry. The generated LMD files were analyzed using an in-house developed “mature antigen-based exclusion” approach adapted from Tembhare et al. [6]. Supplementary Fig. 1B details our sequential gating strategy for leukemia associated immunophenotype (LAIP) identification. A cluster of over 30 events with aberrant immunophenotype was considered for MRD quantification. The sensitivity of our MRD assay was 0.003% with a maximum coefficient of variation of 14.4% (refer to Supplementary Table 1 for our MRD assay validation and formula used for MRD calculation).

**Antigen shift determination**

Differences in expression intensity between baseline and EOI-residual blasts were analyzed for the following antigens (negative & positive controls): CD7 (B-lymphocytes & T-lymphocytes), CD4 (B-lymphocytes and CD4+ T-lymphocytes), CD8 (B-lymphocytes and CD8+ T-lymphocytes), CD5 (B-lymphocytes and T-lymphocytes), CD38 (granulocytes and monocytes). Normalized mean fluorescence intensity (nMFI) for all these antigens was calculated for baseline and EOI-residual blasts as previously described [16].

For mature antigen-based MRD analysis, we analyzed the stability of mature T-cell associated antigens (CD7, CD4, CD8, CD5 and surface CD3) available in our MRD panel (Supplemental Table 1). CD38 was analyzed to assess the stability of this potentially targetable antigen by daratumumab. Stability of CD56 could not be analyzed as both CD56 and CD16 were included in the BV510 fluorochrome of our MRD panel.

**Statistical analyses**

Statistical Package for Social Sciences (version 23, IBM, Armonk, NY) and MedCalc version 14.8.1 were used for statistical tests. For intergroup comparisons, Chi-squared and Mann-Whitney U tests were used. Occurrence of induction failure (≥5% BM blasts at EOI), relapse, and death were considered events. With the date of disease diagnosis as the starting time point, Kaplan-Meier survival analysis was used to determine 2-year rates of overall survival (OS), relapse-free survival (RFS), and event-free survival (EFS). Wilcoxon’s signed-rank test was used to assess differences in the expression intensity for CD4, CD8, CD5, CD7, CD38, and sur-
face-CD3 (sCD3) antigens between leukemic blasts at diagnosis and residual blast at EOI-MRD. The risks incurred by the presence of mediastinal mass, hyperleukocytosis, immunophenotypic subtype of T-ALL, D8BNC status, and EOI-MRD positive status on OS, RFS, and EFS were determined by Cox proportional hazard model (Wald test). All statistical tests were two-tailed and considered significant at \( P \leq 0.05 \).

### RESULTS

Among 306 consecutive treatment naïve ALL patients, 81 (36%) were of T-lineage origin. Of these 81 patients, the frequency of con-T-ALL, ETP-ALL and near-ETP-ALL was 60% (N=49), 21% (N=17) and 19% (N=15), respectively. Table 1 summarizes the clinico-laboratory characteristics of these patient categories.

Irrespective of immunophenotypic sub classification, T-ALL comprised 22% (47/209) and 35% (34/97) of our pediatric and adult ALL patients, respectively. T-ALL subtype specific clinico-laboratory profiles of our pediatric and adult T-ALL patients are presented in Table 2 and compared in Supplementary Table 2.

#### Antigen expression profile

FCM determined antigen expression profiles of all 81 T-ALL patients are presented in Supplementary Fig. 2. The median (range) T-CD5: Bl-C D5 expression ratio among con-T-ALL, near-ETP-ALL and ETP-ALL blasts was 1.83 (0.85–8.56), 3.39 (1.43–8.21) and 16.12 (11.06–59.21), respectively. Among con-T-ALL patients, 26.5% (N=13) had isolated CD4 expression, 10% (N=5) had isolated CD8 expression, dual expression for both CD4 and CD8 was observed in 45% (N=22) patients, and 20% (N=9) of the patients did not express either antigen. Expression frequency for myeloid/stem cell antigens (ETP-ALL vs. near-ETP-ALL patients) was CD117 (47% vs. 7%, \( P=0.011 \), CD34 (82% vs. 80%, \( P=0.865 \)), HLA-DR (53% vs. 21%, \( P=0.073 \), CD13 (53% vs. 20%, \( P=0.055 \), CD33 (47% vs. 73%, \( P=0.131 \)), and CD11b (29% vs. 21%, \( P=0.631 \)).

Differences in the percentage of patients expressing immaturity associated antigens (CD10, CD34, and CD117), B-lineage antigens (CD19 and CD79a), myeloid antigens (CD13, CD11b, CD33), and non-lineage-specific antigens (CD123, CD56, and CD38) among our immunophenotypic

### Table 1. Clinical and laboratory characteristics of T-ALL subcategories.

| Parameters                      | Overall T-ALL (N=81) | T-ALL subcategories | \( P \)          |
|---------------------------------|----------------------|---------------------|------------------|
| **Median (range) age in years** |                      |                     |                  |
| Age group                       | 17 (1–52)            | 15 (1–50)           | 17 (13–39)       | 0.882 0.003 0.016 |
| Pediatric (%)                   | 47 (58)              | 34 (72%)            | 7 (15%)          | 0.003 0.168 0.040 |
| Adult (%)                       | 34 (42)              | 15 (44%)            | 10 (29%)         | 0.003 0.168 0.040 |
| Sex (male:female)               | 3.8:1                | 4.4:1               | 3.2:1            | 1.700 0.465 0.485 |
| Median (range) Hb in g/L        | 90 (30–140)          | 30 (10–140)         | 30 (10–140)      | 0.737 0.008 0.080 |
| Median (range) WBC count, \( \times 10^9/L \) | 171 (13–850)       | 171 (13–850)        | 171 (13–850)     | 0.049 0.005 0.751 |
| Median (range) platelet, \( \times 10^9/L \) | 54 (20–380)         | 73 (20–366)         | 125 (30–290)     | 0.911 0.008 0.080 |
| Median (range) BM blast, %      | 87 (22–99)           | 87 (22–99)          | 87 (22–99)       | 0.473 0.795 0.663 |
| Hyperleukocytosis               | 41%                  | 45%                 | 18%              | 0.034 0.046 0.567 |
| Hepatomegaly                    | 42%                  | 42%                 | 27%              | 0.204 0.283 0.319 |
| Splenomegaly                    | 56%                  | 56%                 | 47%              | 0.516 0.550 0.489 |
| Lymphadenopathy                 | 78%                  | 73%                 | 87%              | 1.000 0.290 0.343 |
| Mediastinal mass                | 31%                  | 36%                 | 33%              | 0.388 0.842 0.095 |
| CNS involvement at diagnosis    | 3.2%                 | 2 (5)               | 0%               | -      0.417 0.499 |
| D8BNC                           | 35%                  | 32%                 | 54%              | 0.223 0.168 0.440 |
| EOI-MRD positive                | 39% (N=59)           | 22.5% (N=49)        | 75% (N=12)       | 0.865 0.001 0.009 |
| Relapse                         | 30% (N=60)           | 18% (N=40)          | 17% (N=12)       | 0.292 0.947 0.204 |
| OS at 24 months                 | 65.2% (N=66)         | 70.6% (N=42)        | 60.4% (N=13)     | 0.130 0.551 0.019 |
| RFS at 24 months                | 76.1% (N=60)         | 80% (N=40)          | 79% (N=12)       | 0.292 0.956 0.190 |
| EFS at 24 months                | 64.5% (N=66)         | 70.3% (N=42)        | 66.6% (N=13)     | 0.076 0.978 0.013 |

Abbreviations: BM, bone marrow; CNS, central nervous system; D8BNC, day 8 blast not cleared; EFS, event-free survival; EOI-MRD, end-of-induction-measurable residual disease; Hb, hemoglobin; N, number of patients analyzed; NA, not applicable; OS, overall survival; PB, peripheral blood; RFS, relapse-free survival; WBC, white blood cells.
Adult patients

T-ALL subtype

| Parameters                  | T-ALL subtype | Adult patients          |
|----------------------------|---------------|-------------------------|
|                           | Con-T-ALL     | ETP-ALL                 |
| Median age (range) in years| 12 (1-18)     | 16 (13-17)              |
| Sex (male:female)          | 3:8:1         | 6:1 (5:1)               |
| Median range (Hb in g/L)   | 91 (30-141)   | 97 (30-131)             |
| Median range (WBC, ×10^9/L)| 110 (1.9-450) | 90.4 (3.2-267)         |
| Median range (platelet, ×10^9/L) | 83 (22-366) | 125 (30-245)          |
| Median range (BM blast)    | 87 (23-97)    | 86 (22-98)              |
| Median range (PB blast %)  | 84 (2-96)     | 86 (2-98)               |
| Hyperleukocytosis          | 53%           | 29% (67%)               |
| Hepatomegaly               | 45%           | 40% (67%)               |
| Splenomegaly               | 61.3%         | 40% (67%)               |
| Lymphadenopathy            | 75%           | 100% (100%)             |
| Mediastinal mass           | 40%           | 20% (17%)              |
| CNS involvement            | 4%            | 0% (0%)                 |
| Induction death            | 7%            | 0% (0%)                 |
| Induction failure          | 0%            | 0% (0%)                 |
| DBBNC                      | 30%           | 50% (50%)               |
| EOI-MRD positive           | 15%           | 83% (60%)               |
| Relapse                    | 11%           | 17% (17%)               |
| OS at 24 months            | 79%           | 67% (100%)              |
| RFS at 24 months           | 87%           | 83% (75%)               |
| EFS at 24 months           | 81%           | 80% (76%)               |
| ETP-ALL vs. Con-T-ALL      | 0.295         | 0.056 (0.343)           |
| ETP-ALL vs. ETP-ALL        | 0.906         | 0.307 (0.825)           |
| Near-ETP-ALL vs. Con-T-ALL | 0.181         | 0.465 (0.517)           |
| Near-ETP-ALL vs. ETP-ALL   | 0.366         | 0.198 (0.810)           |
| ETP-ALL vs. Con-T-ALL      | 0.731         | 0.175 (0.240)           |
| BM blast %                 | 0.149         | 0.845 (0.029)           |
| PB blast, %                | 0.268         | 0.883 (0.074)           |
| Hyperleukocytosis          | 0.008         | 0.240 (0.533)           |
| Hepatomegaly               | 0.109         | 0.829 (0.476)           |
| Splenomegaly               | 0.463         | 0.370 (0.655)           |
| Lymphadenopathy            | 0.906         | 0.207 (0.207)           |
| Mediastinal mass           | 0.153         | 0.402 (0.286)           |
| CNS involvement            | 0%            | 0% (0%)                 |
| Induction death            | 0%            | 0% (0%)                 |
| Induction failure          | 0%            | 0% (0%)                 |
| DBBNC                      | 0.105         | 0.486 (0.074)           |
| EOI-MRD positive           | 0.346         | 0.001 (0.025)           |
| Relapse                    | 0.060         | 0.060 (0.060)           |
| OS at 24 months            | 0.564         | 0.020 (0.297)           |
| RFS at 24 months           | 0.937         | 0.805 (0.720)           |
| EFS at 24 months           | 0.937         | 0.878 (0.943)           |

Abbreviations: BM, bone marrow; CNS, the central nervous system; DBBNC, day 8 blast not cleared; EFS, event-free survival; EOI-MRD, end-of-induction-measurable residual disease; Hb, hemoglobin; N, number of patients analyzed; NA, not applicable; OS, overall survival; PB, peripheral blood; RFS, relapse-free survival; WBC, white blood cells.

EOI-MRD assessment

Among 60 patients who completed induction, EOI-MRD was tested in 59 (40 con-T-ALL, 12 ETP-ALL, and 7 near-ETP-ALL). A median of 2.3 million events (range, 0.18 to 7.3 million) was acquired for analysis, and over 1.5 million events were acquired in 68% of the samples. EOI-MRD was positive in 39% of the samples tested (32% of pediatric and 52% of adult samples). EOI-MRD was frequently positive among ETP-ALL (75%, P=0.001) and near-ETP-ALL (71%, P=0.009) patients, compared to con-T-ALL patients (22.5%).

Median (range) MRD quantified among con-T-ALL, ETP-ALL, and near-ETP-ALL samples was 0.192% (0.015–2.125), 5.360% (0.125–30.306), and 4.250% (0.532–10.436), respectively. There was a significant difference in EOI-MRD quantified between con-T-ALL vs. near-ETP-ALL patients (P=0.019), but not between con-T-ALL vs. ETP-ALL (P=0.074) and ETP-ALL vs. near-ETP-ALL (P=0.898) patients. Comparison of the clinicos-laboratory profiles of our EOI-MRD positive and negative T-ALL patients, and their 2-year OS, RFS, and EFS rates are presented in Supplementary Table 3.

Age group-specific analysis revealed significantly different frequencies of EOI-MRD positivity between the subcategories of T-ALL (con-T-ALL vs. ETP-ALL vs. near-ETP-ALL) among pediatric (15% vs. 83.3% vs. 60%, P=0.002), but not adult T-ALL patients (38.5% vs. 67% vs. 100%, P=0.190).
Antigen stability

Analysis of the effect of induction therapy on expressions of CD4, CD8, CD7, CD5, CD38, and sCD3 antigens revealed statistically significant upregulations of CD8 ($P=0.046$) and CD38 ($P=0.046$) expression in EOI blasts of con-T-ALL and ETP-ALL patients, respectively (Supplementary Fig. 3).

OS, RFS, and EFS

Among the 81 T-ALL patients, 4 died before treatment and 11 left hospital care before initiating treatment (refer to Supplementary Fig. 4 for disease course during follow-up). Among the remaining 66 patients who were treated, 6 died during the induction phase (4.7% of con-T-ALL, 7.6% of ETP-ALL, and 27% of near-ETP-ALL; $P=0.068$). Induction failure was observed in 8.3% of ETP-ALL, 30% of near-ETP-ALL, and none of our con-T-ALL patients ($P=0.005$). A mean ($±$SD) follow-up of 12 ($±$10) months was available among 60 patients who had completed induction. None of the patients underwent hematopoietic stem cell transplantation.

Irrespective of the age at diagnosis and immunophenotypic subclassification, 2-year OS, RFS, and EFS rates among our T-ALL patients were 65%, 76%, and 64.5%, respectively. The survival profiles of our T-ALL patients pertinent to their immunophenotypic subcategorization are depicted in Table 1 and Fig. 2. Expression of CD56, CD19, and CD79a in the blasts did not have any significant impact ($P>0.05$) on the OS, RFS, and EFS among any of the immunophenotypic subcategories of T-ALL. The 2-year OS, RFS, and EFS specific to our pediatric and adult patients stratified by T-ALL subtypes are summarized in Table 2 and compared in Supplementary Table 2.

Impact of EOI-MRD status on survival

Irrespective of immunophenotypic subclassification and age, there were significant differences in 2-year OS (86% vs. 48%, $P=0.013$), RFS (87% vs. 57%, $P=0.022$), and EFS (83% vs. 58%, $P=0.008$) between our EOI-MRD negative vs. positive T-ALL patients (refer to Supplementary Table 3 and Supplementary Fig. 5A for Kaplan-Meier survival curves).

Age group specific analysis revealed significant differences in 2-year OS (95% vs. 53%, $P=0.044$), RFS (95% vs. 60%, $P=0.010$), and EFS (95% vs. 60%, $P=0.010$) among EOI-MRD negative vs. positive pediatric T-ALL patients. However, 2-year OS (58% vs. 41%, $P=0.303$), RFS (67% vs. 54%, $P=0.727$) and EFS (50% vs. 44%, $P=0.435$) was not significantly different between EOI-MRD negative vs. positive adult T-ALL patients (refer to Supplementary Fig. 5B for Kaplan-Meier survival curves).

T-ALL subtype specific analysis revealed significant differ-
ence in 2-year OS (94% vs. 37%, \(P=0.012\)), RFS (94% vs. 50%, \(P=0.005\)), and EFS (94% vs. 50%, \(P=0.005\)) between EOI-MRD negative vs. positive pediatric con-T-ALL patients. However, there were no significant differences in 2-year OS (50% vs. 53%, \(P=0.874\)), RFS (62% vs. 66%, \(P=0.584\)) and EFS (42% vs. 53%, \(P=0.891\)) between the EOI-MRD negative vs. positive adult con-T-ALL patients (refer to Supplementary Fig. 5B for Kaplan–Meier survival curves).

The lower number of patients available at the EOI timepoint in ETP-ALL (6 pediatric and 6 adult) and near-ETP-ALL (5 pediatric and 2) subtypes precluded analysis of age group specific impact of EOI-MRD status on survival in these categories.

Cox proportional hazard regression analysis was performed to identify risks incurred by the immunophenotypic subtype of T-ALL, presence of mediastinal mass and hyperleukocytosis at diagnosis, and D8BNC and EOI-MRD positive status on 2-year OS, RFS, and EFS on our pediatric and adult T-ALL patients.

Fig. 2. Two-year overall survival (OS), relapse-free survival (RFS), and event-free survival (EFS) across all immunophenotypic subcategories of T-ALL analyzed together among all age groups (first row), pediatric patients (second row), and adult patients (third row).
2 years-RFS

P
95% CI
95% CI
P

2 years-OS
HR
95% CI
P

(\(P=0.039\)) in our cohort. ET P-ALL comprised 29% of T-ALL patients is similar to previously observed frequencies of 11% and 14% [4, 6]. As reported in other studies [17, 18], we too observed adult age predilection for ETP-ALL. The 13% frequency of near-ETP-ALL is similar to the 33% frequency observed by Van Vlierberghe et al. [19]. The 13% frequency of near-ETP-ALL is considered under a common ETP-ALL category. As known, as only a few studies have recognized this entity [3, 4, 6, 19]. In the present study, near-ETP-ALL was also frequent among adult T-ALL patients (26%, \(P=0.040\)). This frequency is similar to the 33% frequency observed by Van Vlierberghe et al. [19]. The 13% frequency of near-ETP-ALL we observed is similar to the 17% frequency reported by the Children’s Oncology Group [4], but is higher than the 5.4% frequency reported by Tembhare et al. [6] from India. Our results indicate that these immunophenotypic subcategories of T-ALL cannot be distinguished by the presence of hepatosplenomegaly, lymphadenopathy, or mediastinal mass at diagnosis (\(P>0.05\)). Regarding the laboratory parameters at diagnosis, our adult ETP-ALL patients had significantly lower white blood cell (\(P=0.036\)) and higher platelet (\(P=0.016\)) counts compared to those in our adult control T-ALL patients. These findings are consistent with those of prior studies [21, 22]. However, Ma et al. [23] had observed significantly low white blood cell counts among their pediatric ETP-ALL patients.

### DISCUSSION

#### Demography

The 15% frequency of ETP-ALL documented in our pediatric T-ALL patients is similar to previously observed frequencies of 11% and 14% [4, 6]. As reported in other studies [17, 18], we too observed adult age predilection for ETP-ALL (\(P=0.039\)) in our cohort. ETP-ALL comprised 29% of our adult T-ALL patients. The documented frequency is variable, with rates of 17% in the United States, 32% in Germany, and 47% in China [1, 18-22]. This marked heterogeneity might be due either to ethnic predisposition or incongruencies in FCI analysis, where both ETP-ALL and near-ETP-ALL are considered under a common ETP-ALL category. The exact worldwide frequency of near-ETP-ALL is unknown, as only a few studies have recognized this entity [3, 4, 6, 19]. In the present study, near-ETP-ALL was also frequent among adult T-ALL patients (26%, \(P=0.040\)). This frequency is similar to the 33% frequency observed by Van Vlierberghe et al. [19]. The 13% frequency of near-ETP-ALL we observed is similar to the 17% frequency reported by the Children’s Oncology Group [4], but is higher than the 5.4% frequency reported by Tembhare et al. [6] from India. Our results indicate that these immunophenotypic subcategories of T-ALL cannot be distinguished by the presence of hepatosplenomegaly, lymphadenopathy, or mediastinal mass at diagnosis (\(P>0.05\)). Regarding the laboratory parameters at diagnosis, our adult ETP-ALL patients had significantly lower white blood cell (\(P=0.036\)) and higher platelet (\(P=0.016\)) counts compared to those in our adult control T-ALL patients. These findings are consistent with those of prior studies [21, 22]. However, Ma et al. [23] had observed significantly low white blood cell counts among their pediatric ETP-ALL patients.

#### Immunophenotype at diagnosis

Both ETP-ALL and near-ETP-ALL blasts are proposed to have originated from BM-derived early thymic precursor (ETP) cells that migrated to the thymus. These ETP cells are too immature and have a transcriptome profile enabling differentiation towards T, myeloid, and dendritic cell lineages [1]. The dendritic-lineage orientation of ETP-ALL and near-ETP-ALL blasts was reflected in our results, as we observed a high frequency of CD123 positivity (\(P<0.001\)) in these patients as compared to con-T-ALL patients (Fig. 1). In our T-ALL cohort, CD73 expression was restricted only to ETP-ALL and near-ETP-ALL blasts (\(P=0.007\)). CD86 was significantly expressed only among ETP-ALL blasts (\(P<0.001\)). This contrast might reflect the smaller population of these patients as compared to con-T-ALL patients (Fig. 1). The diagnostic relevance of this observation has to be verified in a larger cohort.

Regarding cross-lineage antigen expression among T-ALL blasts, expression of the CD56 antigen of natural killer (NK) cells is frequently associated with ETP-ALL blasts and confers a poor prognosis [24-27]. Consistent with the literature, we also observed a higher frequency of CD56 expression in our ETP-ALL and near-ETP-ALL patients (\(P=0.009\)). However, in contrast to the available literature, expression of CD56 did not make any difference in the OS, EFS, and RFS of any immunophenotypic subcategories of our T-ALL patients (\(P>0.05\)). This contrast might reflect the smaller cohort size, differences in treatment protocols used, and limited follow-up available among our patients. Despite recent observations regarding the expression of B-lineage markers CD19 and CD79a in ETP-derived blasts [6, 28], the prognostic relevance of such aberrant expression is unknown. In the current study, CD19 expression was observed only among

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**Table 3. Univariate analysis of covariates with event-free, relapse-free, and overall survivals.**

| Variables | 2 years-EFS | 2 years-RFS | 2 years-OS |
|-----------|-------------|-------------|------------|
|           | HR  | 95% CI | \(P\) | HR  | 95% CI | \(P\) | HR  | 95% CI | \(P\) |
| Pediatric | D8BNC | 0.656 | 0.191-3.620 | 0.629 | 0.816 | 0.135-4.924 | 0.825 | 0.311 | 0.035-2.801 | 0.298 |
|          | Mediastinal mass | 1.849 | 0.358-9.549 | 0.463 | 3.109 | 0.347-27.843 | 0.311 | 1.578 | 0.285-8.721 | 0.601 |
|          | Hyper-leucocytosis | 0.422 | 0.082-2.179 | 0.303 | 0.693 | 0.116-4.154 | 0.688 | 0.228 | 0.027-1.951 | 0.117 |
|          | EOI-MRD positive | 10.153 | 1.132-91.096 | 0.038 | 10.081 | 1.123-90.059 | 0.039 | 7.381 | 0.757-71.952 | 0.085 |
|          | ConT-ALL subtype | 1.129 | 0.219-5.821 | 0.885 | 0.701 | 0.117-4.197 | 0.698 | 2.272 | 0.265-19.490 | 0.454 |
|          | ETP-ALL subtype | 0.887 | 0.103-7.132 | 0.887 | 1.227 | 0.137-10.984 | 0.855 | 0.932 | 0.108-8.014 | 0.949 |
|          | Near-ETP-ALL subtype | 0.956 | 0.115-7.947 | 0.967 | 1.425 | 0.159-12.757 | 0.762 | 0.780 | 0.091-6.700 | 0.821 |
| Adult    | D8BNC | 1.166 | 0.326-4.172 | 0.814 | 0.448 | 0.046-4.336 | 0.488 | 1.456 | 0.388-5.462 | 0.577 |
|          | Mediastinal mass | 3.000 | 0.782-11.502 | 0.109 | 2.210 | 0.426-11.462 | 0.311 | 5.008 | 1.029-24.374 | 0.056 |
|          | Hyper-leucocytosis | 1.784 | 0.615-5.178 | 0.287 | 4.084 | 0.908-18.368 | 0.067 | 1.482 | 0.483-4.547 | 0.491 |
|          | EOI-MRD positive | 1.648 | 0.461-5.883 | 0.442 | 1.302 | 0.291-5.828 | 0.730 | 2.024 | 0.501-8.185 | 0.323 |
|          | ConT-ALL subtype | 0.425 | 0.144-1.253 | 0.121 | 0.607 | 0.135-2.738 | 0.516 | 0.361 | 0.117-1.117 | 0.077 |
|          | ETP-ALL subtype | 0.730 | 0.203-2.623 | 0.630 | 0.461 | 0.055-3.833 | 0.473 | 0.824 | 0.226-3.002 | 0.769 |
|          | Near-ETP-ALL subtype | 7.995 | 2.000-31.968 | 0.003 | 11.122 | 1.533-80.779 | 0.017 | 6.649 | 1.891-23.383 | 0.003 |

Abbreviations: CI, confidence interval; D8BNC, day 8 blast not cleared; EFS, event-free survival; EOI-MRD, end-of-induction-measurable residual disease; HR, hazard ratio; OS, overall survival; PB, peripheral blood; RFS, relapse-free survival.
and CD38 (P=0.046) in the EOI blasts of con-T-ALL and down-regulated during treatment [29]. T-ALL MRD analysis foolproof as these immaturity-related antigens are frequently their precursors that can mimic residual disease [6]. Due by FCM is also hindered by the presence of NK cells and expressing lymphocytes [4, 8, 9, 29]. This approach is not like CD34, TdT, and CD99 on CD7 and cytoplasmic CD3 identifying the expression of immaturity associated markers to these shortcomings, most T-ALL MRD data are by high-throughput sequencing for IgG and TCR rearrangements [3].

ETP-ALL and near-ETP-ALL blasts and not among con-T-ALL blasts (P=0.004). In contrast, CD79a expression was not predilected towards any of the immunophenotypic subcategories of T-ALL (P=0.172). Importantly, aberrant expression of either CD19 or CD79a did not translate into inferior 2-year survival outcomes in our con-T-ALL, ETP-ALL, and near-ETP-ALL patient categories (P>0.05).

EOI-MRD

Traditional T-ALL MRD assessment by FCM relies on identifying the expression of immaturity-associated markers like CD34, TdT, and CD99 on CD7 and cytoplasmic CD3 expressing lymphocytes [4, 8, 9, 29]. This approach is not foolproof as these immaturity-related antigens are frequently down-regulated during treatment [29]. T-ALL MRD analysis by FCM is also hindered by the presence of NK cells and their precursors that can mimic residual disease [6]. Due to these shortcomings, most T-ALL MRD data are by high-throughput sequencing for IgG and TCR rearrangements. The literature on T-MRD by FCM is limited [4, 8, 9, 29, 30]. However, these molecular MRD detection techniques might not be successful in ETP-ALL and near-ETP-ALL samples as these leukemias originate from precursor cells that are too immature to have undergone TCR rearrangement [3].

With the increased availability of ≥8 color flow cytometers, the results and sensitivity of T-MRD assessment by FCM are highly comparable to molecular T-MRD assays [8]. Use of 8–9 color panels by the Children’s Oncology Group (COG) yielded EOI-MRD detection rates of 30.5%, 81.4%, and 64.8% in pediatric (N=1,144) con-T-ALL, ETP-ALL, and near-ETP-ALL patients, respectively [4]. In an Indian study in which 35 T-ALL patients of all age groups were analyzed using an 8-color panel, EOI-MRD was detectable in 37% of the patients [30]. A recent study discussed the experience with T-ALL MRD using an 11-antigen 10-color FCM panel. Use of a mature T-cell antigen-based “exclusion” approach for gating detected EOI-MRD in 46.5% of the pediatric T-ALL cohort (N=269) [6]. In all these studies, cumulative MRD positivity observed among ETP-ALL and near-ETP-ALL patients was higher than the MRD positivity observed among con-T-ALL patients (74% with P<0.001 [6], 73.1% by the COG [4], and 67% with P=0.033 [30]). Our result was similar (73% with P<0.001).

Tembhare et al. [6] observed subdued down-regulation of surface CD3, CD4, CD5, CD8, and CD38 expression in their EOI-residual blasts. In the current study, we observed stable expression of CD4, CD5, CD7, and surface CD3 between EOI-residual leukemic blasts (P=0.05) among all immunophenotypic subcategories of T-ALL. However, there was upregulated expression of CD8 (P=0.046) and CD38 (P=0.046) in the EOI blasts of con-T-ALL and ETP-ALL samples, respectively (Supplementary Fig. 3). Tembhare et al. [6] described that these immunophenotype shifts were too subtle to interfere with MRD analysis. Our findings are similar. These collective results indicate that the mature T-cell-related antigen-based approach is reliable for T-MRD analysis by FCM. Importantly, the expression of CD38 in nearly 98% of T-ALL patients at diagnosis in the present and previous [6] studies, along with the stable/upregulated expression of this antigen in the EOI-residual blasts, can be used as a potential target for daratumumab therapy [31]. In our cohort, CD73 expression was specifically associated with ETP-ALL and near-ETP-ALL patients (Fig. 2). Recent optimistic results were obtained with the use of anti-CD73 monoclonal antibody-based treatment in solid tumors [32]. Validation of the stability and specific expression of CD73 in ETP-ALL and near-ETP-ALL patients in a larger cohort would provide a rationale for initiating anti-CD73-based clinical trials in the treatment of these patients.

EOI-MRD status among our ETP-ALL and near-ETP-ALL patients was independent of their baseline clinical and laboratory characteristics. However, our EOI-MRD negative con-T-ALL patients were frequently diagnosed with mediastinal mass, high white blood cell count, and hyperleucocytosis at diagnosis as compared to those in the EOI-MRD positive counterparts.

Outcome in pediatric patients

Conter et al. [33] observed D8BNC status among 55% of their pediatric ETP-ALL patients. This result is similar to that in our cohort, where 50% of patients in each of the ETP-ALL and near-ETP-ALL categories were steroid unresponsive on day 8 (Table 2). High incidence of induction failure and disease relapse has been documented among pediatric ETP-ALL patients [2, 4, 23, 34]. However, none of our pediatric con-T-ALL, ETP-ALL, or near-ETP-ALL patients experienced induction failure or had a significantly higher relapse (Table 2). These contrasting observation have to be validated in a larger cohort with extended follow-up.

We observed no differences (P>0.05) in the 2-year OS, RFS, and EFS among our pediatric con-T-ALL (79%, 83%, and 81%, respectively), ETP-ALL (67%, 87%, and 80%), and near-ETP-ALL (100%, 75%, and 75%) patients. Consistently, there were no significant differences in 5-year OS and EFS among the con-T (92% and 86.9%, respectively), ETP-ALL (93% and 87%), and near-ETP-ALL (91.6% and 84.4%) patients documented in the largest ever pediatric T-ALL data by the Children’s Oncology Group [4]. However, inferior 2-, 4-, 5-, and 10-year OS and EFS have also been documented among pediatric ETP-ALL patients from Italy, Japan, China, and USA, respectively [2, 23, 34]. These different observations might be due to heterogeneity in the sample sizes and ethnicities of the study cohorts. Hence, the true prognostic relevance of both ETP-ALL and near-ETP-ALL will be determined only by prospective studies with a large sample size and longer follow-up.

Irrespective of immunophenotypic sub-classification, our EOI-MRD negative pediatric T-ALL patients had significantly better 2-year OS (95% vs. 53%, P=0.044), RFS (95% vs. 60%, P=0.010), and EFS (95% vs. 72%, P=0.010) than their EOI-MRD positive counterparts, consistent with previous observations [7].
Outcome in adult patients

In the current study, we did not observe any differences in 2-year OS, EFS, and RFS between our adult con-T-ALL and ETP-ALL patients (Table 2). These findings are consistent with two prior studies [21, 22], but discordant with the inferior OS documented among adult & adolescent ETP-ALL patients by Jain et al. [20]. Importantly, our adult near-ETP-ALL patients had the most inferior 2-year OS, EFS, and RFS as compared to that in the con-T-ALL and ETP-ALL patients diagnosed in this age group (Table 2).

An important observation from our study is the EOI-MRD positive status (irrespective of T-ALL subtype) and the presence of near-ETP-ALL immunophenotype (irrespective of EOI-MRD status) influencing the 2-year survival profile among our pediatric and adult T-ALL patients, respectively. These results are encouraging concerning hematopoietic stem cell transplantation at first remission in EOI-MRD positive pediatric T-ALL patients and all adult T-ALL patients diagnosed with near-ETP-ALL. However, the small age-specific sample size in our study precludes any definite conclusions.

CONCLUSION

Both ETP-ALL and near-ETP-ALL are common among adult T-ALL patients. Irrespective of age at diagnosis, both these entities are associated with a high frequency of EOI-MRD positivity. Our results indicate adverse 2-year survival conferred by the presence of EOI-MRD positivity among pediatric T-ALL patients and by the diagnosis of near-ETP-ALL phenotype among adult T-ALL patients. However, large prospective clinical trials are warranted to confirm these conclusions.

Study limitations

Being a relatively rare disease, the number of patients in our cohort might not be powered for exact outcome analysis across each immunophenotypic subtype of T-ALL. Hence, the survival outcomes documented in our study have to be validated in a larger cohort. The mutational profile of leukemic lymphoblasts was not evaluated in our patients. Being retrospective data, our study results are purely observational and did not explore the underlying leukemogenic differences between the subtypes of T-ALL.

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Authors’ Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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Supplementary Fig. 1. (A) Algorithm used in this study to classify T-ALL patients based on flow cytometric immunophenotyping.
Supplementary Fig. 1. (B) Sequential gating for measurable residual disease assessment. Among all events acquired, singlets were gated on forward scatter (FS) peak vs. area contour plot ‘a’. Among these singlets, CD7 positive events (CD7+) were gated on CD7 vs. side scatter (SSC) contour plot ‘b’. These CD7+ events comprise mature T-lymphocytes, NK T/NK cells, dim CD7 expressing myeloid blasts, and leukemia associated immunophenotype events (LAIP) events. From these CD7+ events, surface CD3 (sCD3) positive normal T-lymphocytes (normal T Ly, orange events) and sCD3 negative events (sCD3-CD7+) were separated on sCD3 vs. CD7 contour plot ‘c’. This sCD3-CD7+ population comprising LAIP and NK and T/NK cells were analyzed in cyCD3 vs. CD16+56 plot ‘e’ to segregate NK cells and T/NK cells (greyish-green and black dots, respectively). The remaining cyCD3 positive, CD56 & CD16 negative events are our suspected LAIP (labeled as ‘??’). The distribution of CD4 vs. CD8 and CD5 vs. CD38 expression among NK cells and T/NK cells were analyzed in scatter plots ‘f’ and ‘h’. The suspected LAIP events (‘??’ gate) were analyzed in CD5 vs. CD38 dot plot ‘g’ and were divided into target 1 (CD5 positive & CD38 negative), target 2 (both CD5 and CD38 positive), and ETP-ALL target (CD5 negative and CD38 positive or negative). The CD5 and CD38 expression limits in plot ‘g’ were set based on the CD38 and CD5 expression among NK cells and T/NK cells in plot ‘h’. The ‘??’ events falling within each of these target gates in plot ‘g’ were analyzed in isolation for their CD7 vs. CD34 expression in dot plots ‘i’, ‘j’, and ‘k’, followed by their CD4 vs. CD8 expression in dot plots ‘l’, ‘m’, and ‘n’. The ‘??’ events that were showing normal, i.e., mutually exclusive distribution for CD4 and CD8 (in ‘i’, ‘m’, and ‘n’ dot plots) were not considered as LAIP and were considered to be normal mature T-lymphocytes that have deprived their sCD3 during processing. In this example of con-T-ALL-MRD, the ‘??’ events had a moderate expression for both CD5 and CD38 (target 2), was negative for CD34 expression (plot ‘k’), and had negative to dim expression for both CD4 and CD8 (plot ‘n’). These events were quantified as LAIP events (red dots). The tight clustering of these LAIP events was ensured in FSC vs. SSC plot ‘o’. The antigen expression profile of this LAIP cluster was further compared among all the singlet gated events in various immunophenotype combinations across plots ‘p’ to ‘v’. In samples that were MRD negative by this approach, two modifications were made in the gating strategy. First, the sCD3-CD7+ gate in plot ‘c’ was extended further in the y-axis to include more sCD3 dim to moderate and CD7 bright events. This was to ensure that an sCD3 expressing/upregulated LAIP was not missed. Second, the ‘??’ gate in plot ‘e’ was extended further in the x-axis to include more cyCD3+ & CD16+ CD56 positive events. This was done to avoid missing any CD56/CD16 expressing/upregulated LAIP.

Abbreviations: Con-T-ALL, conventional T-ALL; ETP-ALL, early-T-cell acute lymphoblastic leukemia.
Supplementary Fig. 2. Overview of antigen expression profile among the immunophenotypic subtypes of T-ALL patients analyzed.
Supplementary Fig. 3. Box and whisker plots comparing changes in the intensity of CD4, CD8, CD7, CD5, CD38, and sCD3 expression between baseline and end-of-induction residual blasts.

Abbreviations: First row, con-T-ALL patients; second row, ETP-ALL patients; third row, near-ETP-ALL patients.
Supplementary Fig. 4. Follow-up algorithm of patients included in the study.
Supplementary Fig. 5. (A) Kaplan-Meier survival plots depicting the impact of EOI-MRD status on 2-year OS, RFS, and EFS across all T-ALL subtypes. (B) Kaplan-Meier survival plots for 2-year OS, RFS, and EFS among pediatric and adult T-ALL patients concerning their EOI-MRD status.
Supplementary Fig. 5. Continued.
### Supplementary Table 1A. Flow cytometric immunophenotyping panel for acute leukemia diagnosis.

| Tube | BV510 | BV421 | FITC | PE | ECD | PC5.5 | PC7 | APC | APCEF700 | APCEF750 |
|------|-------|-------|------|----|-----|-------|-----|-----|----------|----------|
| 1    |       |       |      |    |     |       |     |     | CD45     | J.33     |
| Clone |       |       |      |    |     |       |     |     |           |          |
| Vendor |       |       |      |    |     |       |     |     |           |          |
| 2    | CD20  | CD123 | CD58 | CD86| CD73| CD34  | CD10| CD19| CD45     | CD45     |
| Clone | 2H7   | 9F5   | 9ICD58| FLN-1| AD2 | 8G12  | ALB1| J3-119| CD19     | CD45     |
| Vendor | BD    | BD    | BC   | BC | BD  | BD    | BC  | BC  | BC       | BC       |
| 3    |       |       |      |    |     |       |     |     |           |          |
| Clone | L243  | YB5.B8| 80H5 | S11D1| J3-119| 8G12 | N901(HLDA6)| 8H8.1| J.33     | BEAR1    |
| Vendor | BD    | BD    | BC   | BC | BC  | BC    | BC  | BC  | BC       | BC       |
| 4    | HLA-DR| CD36  | CD14 | CD123| CD64| CD33  | CD117| CD34| CD45     | CD38     |
| Clone | L243  | FA6.152| RMO52| 9F5 | 22  | D3HL6.0.251| 104D2D1| 581  | J.33     | B9.11    |
| Vendor | BD    | BD    | BC   | BC | BC  | BC    | BC  | BC  | BC       | BC       |
| 5    | CD3   | CD5   | CD1a | CD7 | CD34| TCR γδ| CD56 | CD4  | CD45     | CD8      |
| Clone | SK7   | UCHT2 | BL6  | 8H8.1| 581 | IMMUS510| N901(HLDA6)| 13B8.2| J.33     | B9.11    |
| Vendor | BD    | BD    | BC   | BC | BC  | BC    | BC  | BC  | BC       | BC       |
| 6    |       |       |      |    |     |       |     |     |           |          |
| Clone | YB5.B8| CLB-PO1| HM47 | UCHT1| SJ10.1H11| 581 | J.33 | BEAR1    |          |
| Vendor | BD    | BC    | BC   | BC | BC  | BC    | BC  | BC  | BC       | BC       |

Abbreviations: BC, Beckman Coulter; BD, Beckton Dickinson Lifesciences; Cyto, cytoplasmic antigen.

### Supplementary Table 1B. Panel for minimal residual disease assessment.

| Tube | BV510 | BV421 | FITC | PE | ECD | PC5.5 | PC7 | APC | APCEF700 | APCEF750 |
|------|-------|-------|------|----|-----|-------|-----|-----|----------|----------|
| 1    | CD16 & CD56| Surface CD3| CD8 | CD7 | Cyto CD3 | CD34 | CD5 | CD4 | CD45 | CD38    |
| Clone | 3G8 & HCD56| UCHT1 | B9.11 | 8H8.1 | UCHT1 | 8G12 | BL1a | 138B2 | J.33 | LS198-4-3 |
| Vendor | BL | BD | BC | BC | BC | BC | BC | BC | BC | BC |
| 2    |       |       |      |    |     |       |     |     |           |          |
| Clone |       |       |      |    |     |       |     |     |           |          |
| Vendor |       |       |      |    |     |       |     |     | Invitrogen | BC |

Abbreviations: BC, Beckman Coulter; BD, Beckton Dickinson Lifesciences; BL, BioLegend; Cyto, cytoplasmic antigen.
**MRD % calculation**

Nucleated cells in the processed bone marrow sample were determined in a separate tube (tube 2 of our MRD panel) using cell-permeant nucleic acid binding Syto13 dye. The formula used for MRD quantification is shown below.

\[
\text{Tube 1 LAIP events} \times \frac{\text{Syto13 positive Singlets\%}}{\text{Tube 1 Singlet events}} \times \frac{\text{Syto13 positive CD7 events\%}}{\text{Tube 1 CD7 events\%}} \times 100 = \text{MRD\%}
\]

Abbreviation: LAIP, leukemia associated immunophenotype.

**MRD assay validation**

For lower limit of blank (LLOB) determination, six control samples (non-T-ALL) were processed with our T-MRD panel and one million events were acquired per sample. The mean (±SD) LOB calculated from these four samples was 4.2 (0.95) events. The lowest limit of detection was calculated as 7 events (LLOB+3 times SD of LLOB in control samples), and was rounded off to a cluster of 10 events (0.001%). By spiking assays (treatment naïve T-ALL samples spiked in non-T-ALL control samples) performed in triplicates, we could establish a lowest limit of quantification (LLOQ) as 30 leukemic events in one million acquired events [maximum coefficient of variation (CV) of 11%]. This corresponds to MRD sensitivity of 0.003% with a maximum CV of 14.4%. The table provides dilution experiment results used to calculate LLOQ.

### Supplementary Table 1C. Dilution experiments for lower limit of detection calculation.

| Dilution experiment | Acquired events | LAIP events | % MRD | LAIP events | % MRD |
|---------------------|----------------|-------------|-------|-------------|-------|
|                     | Mean | SD | CV (%) | Mean | SD | CV (%) |
| For 60 events       | Sample 1 | Processing 1 | 11,23,344 | 65  | 0.005786 | 62  | 4.3 | 6.9 | 0.00555 | 0.00037 | 6.8 |
|                     | Processing 2 | 11,11,446 | 64  | 0.005758 | 63.3 | 4.7 | 7.4 | 0.00584 | 0.00061 | 10.5 |
|                     | Processing 3 | 11,14,246 | 57  | 0.005116 | 65  | 4.7 | 7.4 | 0.00584 | 0.00061 | 10.5 |
|                     | Sample 2 | Processing 1 | 11,12,026 | 65  | 0.005845 | 63.3 | 4.7 | 7.4 | 0.00584 | 0.00061 | 10.5 |
|                     | Processing 2 | 11,08,604 | 58  | 0.005232 | 67  | 0.006465 | 11.0 | 3.5 | 11.0 | 0.00305 | 0.00044 | 14.4 |
|                     | Processing 3 | 10,36,383 | 67  | 0.006465 | 11.0 | 3.5 | 11.0 | 0.00305 | 0.00044 | 14.4 |
| For 30 events       | Sample 1 | Processing 1 | 10,90,239 | 28  | 0.002568 | 31.6 | 3.5 | 11.0 | 0.00305 | 0.00044 | 14.4 |
|                     | Processing 2 | 10,19,365 | 32  | 0.003139 | 35  | 0.003447 | 11.0 | 3.5 | 11.0 | 0.00305 | 0.00044 | 14.4 |
|                     | Processing 3 | 10,15,515 | 35  | 0.003447 | 32.6 | 2.08 | 6.38 | 0.00318 | 0.00022 | 7.0 |
|                     | Sample 2 | Processing 1 | 10,45,155 | 32  | 0.003062 | 32.6 | 2.08 | 6.38 | 0.00318 | 0.00022 | 7.0 |
|                     | Processing 2 | 10,17,746 | 35  | 0.003439 | 35  | 0.003447 | 11.0 | 3.5 | 11.0 | 0.00305 | 0.00044 | 14.4 |
|                     | Processing 3 | 10,18,950 | 31  | 0.003042 | 32.6 | 2.08 | 6.38 | 0.00318 | 0.00022 | 7.0 |

Abbreviations: CV, coefficient of variation; LAIP, leukemia associated immunophenotype; MRD, minimal residual disease.
### Supplementary Table 2. Comparison of clinical and laboratory characteristics between adult and pediatric patients among each subtype of T-ALL.

| Parameters                        | All categories (N=81) | Con-T-ALL (N=49) | ETP-ALL (N=17) | Near-ETP-ALL (N=15) |
|-----------------------------------|-----------------------|------------------|----------------|---------------------|
|                                   | Adult (N=34)          | Pediatric (N=47) | Adult (N=15)   | Pediatric (N=34)    | Adult (N=10) | Pediatric (N=7) | Adult (N=9) | Pediatric (N=6) |
| Sex (male:female)                 | 3.2:1                 | 4.2:1            | 2.3:1          | 6.1:1               | 6.3          | 5.1             | 6.3          | 5.1               |
| Median (range)                    | 85.6 (61-142)         | 89 (63-142)      | 80 (61-128)    | 88 (69-133)         | 88           | 83              | 88           | 83               |
| HB in g/L                         | 90 (30-141)           | 91 (30-141)      | 97 (30-131)    | 83 (41-129)         | 83           | 45              | 83           | 45               |
| Median (range)                    | 70.3 (1-480)          | 110 (1.1-349)    | 55 (1-480)     | 68 (1-480)          | 244          | 272             | 244          | 272              |
| WBC count, ×10⁹/L                 | 85.6 (61-142)         | 90 (30-141)      | 80 (61-128)    | 83 (41-129)         | 83           | 45              | 83           | 45               |
|                  | (1.9-850)            | (1.9-850)        | (1.9-850)      | (1.9-850)           | (3.6-131)    | (3.5-90)        | (3.6-131)    | (3.5-90)          |
| Hb, median (range)                | 85.6 (61-142)         | 90 (30-141)      | 80 (61-128)    | 83 (41-129)         | 83           | 45              | 83           | 45               |
|                  | (1.9-850)            | (1.9-850)        | (1.9-850)      | (1.9-850)           | (3.6-131)    | (3.5-90)        | (3.6-131)    | (3.5-90)          |
| Hyperleukocytosis (%)             | 27%                   | 27%              | 20%            | 14%                 | 16%          | 5%              | 14%          | 5%               |
| Lymphadenopathy (%)               | 75%                   | 59%              | 40%            | 16%                 | 17%          | 0%              | 17%          | 0%               |
| Mediastinal mass (%)              | 27%                   | 29%              | 40%            | 14%                 | 16%          | 0%              | 16%          | 0%               |
| Induction death (%)               | 16%                   | 5%               | 14%            | 14%                 | 17%          | 0%              | 17%          | 0%               |
| Induction failure (%)             | 17.4%                 | 0%               | 17%            | 17%                 | 17%          | 0%              | 17%          | 0%               |
| D8BNC (%)                         | 35%                   | 35%              | 30%            | 30%                 | 30%          | 30%             | 30%          | 30%              |
| EOI-MRD positive (%)              | 52%                   | 32%              | 67%            | 67%                 | 67%          | 67%             | 67%          | 67%              |
| Relapse (%)                       | 33%                   | 13%              | 17%            | 17%                 | 17%          | 17%             | 17%          | 17%              |
| OS at 24 months (%)               | 39.8%                 | 79.5%            | 51.4%          | 51.4%               | 48.1%        | 79.3%           | 48.1%        | 79.3%            |
| RFS at 24 months (%)              | 60.4%                 | 84.3%            | 67.6%          | 67.6%               | 64.3%        | 87.3%           | 64.3%        | 87.3%            |
| EFS at 24 months (%)              | 30%                   | 80.3%            | 53.6%          | 53.6%               | 44.5%        | 81.4%           | 44.5%        | 81.4%            |

Abbreviations: BM, bone marrow; CNS, central nervous system; D8BNC, day 8 blast not cleared; EFS, event-free survival; EOI-MRD, end-of-induction-measurable residual disease; Hb, hemoglobin; N, number of patients analyzed; NA, not applicable; OS, overall survival; PB, peripheral blood; RFS, relapse-free survival; WBC, white blood cells.
**Supplementary Table 3.** EOI-MRD status specific clinical and laboratory characteristics across subcategories of T-ALL.

| Parameters                          | All T-ALL subtypes (N = 59) | Con-T-ALL (N = 40) | ETP-ALL (N = 12) | Near ETP-ALL (N = 7) |
|-------------------------------------|-----------------------------|-------------------|------------------|---------------------|
|                                     | MRD- N=36 | MRD+ N=23 (39%) | P | MRD- N=31 | MRD+ N=9 (22.5%) | P | MRD- N=3 | MRD+ N=9 (75%) | P | MRD- N=2 | MRD+ N=5 (71%) | P |
| Median (range)                      | 14 - 23 | 0.001 | | 11 - 22 | 0.015 | | 19 - 17 | 0.727 | | 10 - 18 | 0.190 | |
| age in years                        | (1-31) | (9-50) | | (1-31) | (9-50) | | (15-20) | (13-39) | | (5-15) | (11-34) | |
| Sex (male:female)                   | 6:2 | 0.241 | | 5:2 | 0.672 | | All males: 5.5:1 | 0.371 | | 2:0 | 1.5:1 | 0.290 |
| Median (range)                      | 92 - 88 | 0.963 | | 93 - 85 | 0.799 | | 92 - 97 | 0.727 | | 75 - 75 | 0.857 | |
| Hb in g/L (N=36)                    | (30-142) | (41-135) | | (30-142) | (73-135) | | (66-126) | (61-131) | | (60-90) | (41-90) | |
| WBC count, ×10^9/L (N=36)           | (1.1-736) | (1.0-482) | | (1.1-736) | (9.7-82.5) | | (10.3-480) | (1-267) | | (350-590) | (3-482) | |
| platelet count, ×10^9/L (N=36)      | (20-366) | (20-380) | | (20-366) | (20-95) | | (45-159) | (30-245) | | (44-132) | (32-380) | |
| Median (range)                      | 89 - 65 | 0.199 | | 85 - 62 | 0.241 | | 83 - 45 | 0.582 | | 94 - 82 | 0.571 | |
| BM blast, %                         | (2-98) | (2-99) | | (2-97) | (56-86) | | (80-86) | (2-95) | | (90-98) | (2-99) | |
| Hyperleukocytosis (%)               | 58% | 0.002 | | 58% | 0.002 | | 33% | 0.371 | | 100% | 60% | 0.290 |
| Hepatomegaly (%)                    | 35.3% | 0.519 | | 40% | 0.576 | | 0% | 0.301 | | 50% | 100% | 0.402 |
| Splenomegaly (%)                    | 56% | 0.666 | | 57% | 0.509 | | 33% | 0.778 | | 100% | 75% | 0.576 |
| Lymphadenopathy (%)                 | 73% | 0.133 | | 72% | 0.379 | | 67% | 0.490 | | 100% | 100% | NA |
| Mediastinal widening (%)            | 51.5% | 0.013 | | 55% | 0.003 | | 50% | 0.747 | | 0% | 20% | 0.495 |
| DBBNC (%)                           | 39% | 0.979 | | 39% | 0.629 | | 0% | 0.343 | | 100% | 33% | 0.248 |
| Blasts expressing                   | CD19 | 0.053 | | 0% | 0% | | NA | 0% | 0.248 | | 0% | 40% | 0.290 |
| Surface CD3                         | 14% | 0.076 | | 40% | 0.519 | | 0% | 0.546 | | 50% | 25% | 0.540 |
| CD79a                               | 14% | 0.142 | | 10% | 0% | | NA | 0% | 0.157 | | 0% | 20% | 0.495 |
| CD56                                | 11% | 0.028 | | 4/31 | 0.156 | | 0/3 | 0.371 | | 0/2 | 3/5 | 0.147 |
| OS at 24 months                     | 85.7% | 0.013 | | 83.2% | 0.017 | | 100% | 77.8% | 0.395 | | 100% | 50% | 0.264 |
| RFS at 24 months                    | 87.2% | 0.022 | | 85.2% | 0.125 | | 100% | 72.9% | 0.445 | | 100% | 26.7% | 0.155 |
| EFS at 24 months                    | 83% | 0.008 | | 80.3% | 0.050 | | 100% | 64.8% | 0.339 | | 100% | 30% | 0.174 |

Abbreviations: BM, bone marrow; CNS, central nervous system; DBBNC, day 8 blast not cleared; EFS, event-free survival; EOI-MRD, end-of-induction-measurable residual disease; Hb, hemoglobin; NA, not applicable; OS, overall survival; PB, peripheral blood; RFS, relapse-free survival; WBC, white blood cell.