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ORIGINAL ARTICLE

Flow cytometric Immunophenotyping and Hematological Findings at Diagnosis and Relapse of Pediatric Acute Lymphoblastic Leukemia Patients

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SUMMARY

Background: **Acute lymphoblastic leukemia (ALL) is a common pediatric leukemia caused by lymphoid precursor proliferation. We analyzed immunophenotyping and hematological findings, as the risk of relapse, of pediatric ALL patients at diagnosis and relapse.**
Methods: **Peripheral blood and bone marrow samples of 30 pediatric ALL patients were collected at diagnosis and at relapse. The latter was evaluated for immunophenotyping and cytochemical staining (Periodic Acid Schiff stain (PAS)), while hematological findings were assessed in the former one.**
Results: **The percentage of PAS-positive patients, TdT, and CD4 expression were significantly higher at diagnosis than relapse ($p = 0.027$, 0.004, and 0.043, respectively), whereas the platelet/lymphocyte ratio (PLR) and neutrophil/lymphocyte ratio were significantly lower at diagnosis ($p = 0.004$ and 0.032, respectively). There were correlations between immunophenotyping and hematology data, including: a) a negative correlation between CD4 expression with blast percentage ($r = -0.927$, $p = 0.003$) and hemoglobin level ($r = -0.991$, $p < 0.001$) at diagnosis and TdT expression with platelet count ($r = -0.441$, $p = 0.017$) at relapse, and b) a positive correlation between CD3 expression with PLR ($r = 0.367$, $p = 0.046$) at relapse.**
Conclusions: **Results suggest that changes in immunophenotyping and hematology findings could be applied as relapse prognostic factors in ALL.**
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KEY WORDS

acute lymphoblastic leukemia, immunophenotyping, relapse, cytochemistry, hematological findings, prognosis

Highlights:

- Some immunophenotyping and hematological changes are significant between diagnosis and relapse in pediatric ALL.

- There is a link between immunophenotyping and hematological findings at diagnosis and relapse in pediatric ALL.
- Some immunophenotyping and hematological findings can have prognostic values in pediatric ALL.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is one of the most common pediatric leukemias with a peak incidence at ages between 2 and 5 years, characterized by the uncontrolled proliferation of lymphoblasts, decreased number of mature lymphocyte cells in the bone marrow (BM) [1,2], and also the unusual presence of blast cells in the peripheral blood (PB) and other organs such as the spleen, liver, central nervous system, and testicles [3]. ALL, as a heterogeneous disorder, has various clinical, morphological, immunophenotypical, genetic, and molecular characteristics [2,4].

It can be diagnosed by flow cytometry immunophenotyping, so that by detecting surface cluster of differentiation (CD) antigens of lymphoblasts [5,6], ALL is classified to different categories such as B- and T-cell lineage ALL [2,4,7]. It has been seen that 20% of ALL cases are of T-cell lineage, 75% of B-cell precursors, and 5% of mature B cells [8]. B-lineage ALL is divided to pro-B (express human leukocyte antigen-DR isotype [HLA-DR], terminal deoxynucleotidyl transferase [TdT], CD34 and CD19), common pre-B (express HLA-DR, TdT, CD34, CD19, CD10, cytoplasmic CD22 [cCD22] and cCD79a), pre-B cμ+ (expresses HLA-DR, CD19, CD10, CD22, and cCD79a), and B-cell ALL (express HLA-DR, CD19, cCD79a, CD20, soluble CD22 [sCD22] and sIgM), and T-lineage ALL to pre-T (express HLA-DR, CD34, TdT, CD7 and cCD3), T-intermediate (express CD7, cCD3, CD2, CD-1a, TdT, and can co-express CD4 and CD8), and T-medullar ALL (CD7, sCD3, CD2, and CD4+ or CD8+) [8,9]. Additionally, some antigens such as myeloid lineage antigens (CD13, CD33, CD117, and/or cytoplasmic myeloperoxidase [cMPO]) possess prognostic values in B- and T-lineage ALL patients [2]. Furthermore, flow cytometry immunophenotyping along with cytochemistry analysis, cytogenetics, and molecular biology is handy in the distinction between acute leukemias [10]. Therapy response of ALL patients is approximately 85%, while 15 - 20% of patients experience relapse following initial chemotherapy [1,11]. Among the most prevalent factors in estimating risk of relapse are some laboratory findings, including early treatment response findings and diagnostic features such as age, white blood cell (WBC) count, immunophenotype, and cytogenetical or molecular abnormalities [1]. Shorter time to relapse, T-cell immunophenotype, older age (age ≥ 10 years), higher WBC count (≥ 50 x 10^9/L) and BM disease are associated with a worse prognosis at relapse [1,12]. The times of relapse differ in T-ALL and B-ALL cases, where relapse in T-ALL occurs during treatment and 6 months after therapy discontinuation, while in B-ALL it could happen > 30 or > 60 months after the diagnosis [1].

Due to the prognostic value of time to relapse, immunophenotyping, and diagnostic features, it seems that their evaluation could be helpful in ALL patients' follow up. This study aimed at determining differences of immunophenotyping and laboratory findings in ALL patients between diagnosis and relapse.

MATERIALS AND METHODS

Patients and samples

In this case series study, PB and BM data of 30 ALL relapse patients from MAHAK's Pediatric Cancer Treatment and Research Center (MPCTRC) in Tehran, collected from April 21, 2010, to August 21, 2018, were analyzed. Diagnosis of ALL was initially based on clinical presentation, morphological, and cytochemical features of leukemic blasts in PB and BM according to the French American British (FAB) criteria and immunological features. All patients had received chemotherapy according to their lineage assignment as established by morphology and immunophenotyping. Inclusion criteria were new cases and pediatric groups presenting with clinical features and abnormal hematological values suggestive of leukemia. Exclusion criteria were patients with prior treatment.

Hematological analysis

Complete blood count (CBC) was performed with peripheral venous blood collected in blood collection tubes containing ethylenediaminetetraacetic acid (EDTA) by a Sysmex hematology analyzer both at diagnosis and relapse. Several parameters were taken into account: WBC, platelet count, hemoglobin (Hb), absolute neutrophil count (ANC), absolute lymphocyte count (ALC), and absolute monocyte count (AMC).

Morphology and cytochemical analysis

The PB and BM aspirate samples were collected in EDTA and heparin-anticoagulation containing tubes, respectively, at the time of diagnosis and relapse and prepared for morphological examination using standard techniques. Samples' smears were stained by Wright-Giemsa to find the presence of blast cells. To exclude cases of acute myeloid leukemia (AML), cytochemical staining with Periodic Acid Schiff (PAS) was performed.

Flow cytometric analysis

Immunophenotypes of leukemic blasts of PB and BM samples were detected by flow cytometry at diagnosis and relapse. At first, samples were incubated with mouse monoclonal antibodies (Dako, Denmark). Then, 10 µL of conjugated monoclonal antibodies with fluorescein isothiocyanate (FITC) (CD7+, HLA-DR+, CD-13+, CD33+, CD10+, CD2+, TdT+, IgM+, cMPO+,

and CD4+) and 10 µL of conjugated antibodies with phycoerythrin (PE) (CD8+, CD5+, CD3+, CD34+, CD-19+, CD20+) were added to the tubes with the same name and 10 µL of peridinin chlorophyll protein (Per-CP) (CD45+) was also poured into all tubes. Afterward, 100 µL of whole blood was incubated with relevant antibodies for 30 minutes at 4°C in a dark place. After incubation, red blood cells were lysed using lysis buffer. Then, the remaining WBCs were washed twice with phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA). The percentage of B-cell lymphoid antigens, T-cell lymphoid antigens, myeloid lineage antigens, and also non-lineage specific antigens TdT+, CD45+, CD34+, and HLA-DR+ (as stem cell/ hematopoietic precursors markers) were immediately measured by Partec Flow Cytometer (Partec PAS, Germany). Data were analyzed with FlowMax software and presented as percent expression of markers (%). Unstained cells in each tube were used as negative controls to set quadrant gates.

Statistical analysis

All statistical analyses were performed using SPSS version 22, and data were expressed as mean ± standard deviation (SD) for variables with normal distribution and median or interquartile range (IQR) for variables deviating from the normal distribution. Also, qualitative data were expressed as frequency and percentage. Normality was assessed by Kolmogorov-Smirnov test. Paired Student's *t*-test and Wilcoxon signed-rank test were used to evaluate the differences between patients at diagnosis and relapse with respect to data that fulfilled normal distribution and for those that did not, respectively. On the other hand, the chi-squared test was used for comparing qualitative variables. Spearman's rho or Pearson's test was used to study the correlation between nonparametric or parametric data. In all the tests, the level of significance was set at $p < 0.05$.

RESULTS

Thirty children with a mean age of 6.7 years (17 male and 13 female) with a ratio male/female of 1.3:1 were enrolled in this study. Of those, 23 patients (76.7%) were B-ALL and 7 patients (23.3%) were T-ALL, as is shown in Table 1.

Comparing immunophenotyping biomarkers and laboratory data at diagnosis and relapse

The percentage of TdT expression was significantly higher at diagnosis than relapse ($p = 0.004$), so that 29 of 30 (96.7%) of TdT expression in ALL patients changed (Figure 1A, B, and C). Seven ALL patients (23.3%) were assessed for CD4 expression and its expression was significantly higher at diagnosis than at relapse ($p = 0.043$) (Figure 1D, E, and F). There was one patient who showed a higher percentage of CD4 expression. We compared the hematological parameters in this patient with other patients who showed a lower percentage of this CD marker and found a patient showing a higher percentage of CD4 expression having a lower Hb level at the time of diagnosis of ALL. In contrast, the percentage of HLA-DR expression, similar to TdT, was slightly higher at diagnosis than at relapse with no significant difference ($p = 0.517$). As is shown in Table 2, a higher percentage of T-cell lymphoid antigens (CD2, CD3, CD5, and CD7) expression was found at relapse relative to diagnosis, but the difference was not statistically significant ($p = 0.469$, 0.168, 0.258 and 0.318, respectively). The percentage of B-cell lymphoid antigens (CD10 and CD19) expression was higher at diagnosis than at relapse with no significant difference ($p = 0.299$ and 0.344, respectively), while the percentage of CD20, the other B-cell lymphoid antigen, was higher at relapse ($p = 0.636$). In addition, compared with the time of diagnosis, there was no significant difference of CD13, CD33, and cMPO expression at relapse ($p = 0.376$, 0.517, and 0.909, respectively) (Table 2).

The comparison of blood counts at diagnosis and relapse showed that the platelet/lymphocyte ratio (PLR) and neutrophil/lymphocyte ratio (NLR) were significantly lower at diagnosis than relapse ($p = 0.004$ and 0.032, respectively) (Figure 1G and H), where one patient showed a higher PLR than other patients at relapse. Subsequently, by comparing other hematological parameters of this patient with other patients, we found that this patient with higher PLR had a lower ALC and lymphocyte/monocyte ratio (LMR) at relapse. Also, the comparison of other hematological parameters in one patient who had a higher NLR than other patients at diagnosis showed a higher ANC at diagnosis. In contrast, as shown in Table 2, the ALC, blast percentage and total WBC count of all patients were higher at diagnosis than relapse with no significant difference ($p = 0.057$, 0.064 and 0.072, respectively). In additional, there was no significant difference in other hematological parameters between diagnosis and relapse.

The comparison of immunophenotyping data and laboratory data with years of relapse in ALL patients demonstrated that years of relapse had a significant, positive association with ANC at diagnosis ($p = 0.028$), so that the risk of relapse in the first year was higher in lower levels of these parameters, while there was no significant association between immunophenotyping and years of relapse. In addition, there was no significant association between type of cells (B- and T-ALL) and years of relapse.

By comparing immunophenotyping and laboratory data with patients' gender, we found that these results were significantly affected by gender. Males had significantly higher NLR at diagnosis than females ($p = 0.009$). Likewise, males had significantly higher CD4 expression, total WBC count, and ANC than females at relapse ($p = 0.042$, 0.029, and 0.047, respectively).

Cytochemistry data were available for 29 of the 30 patients (96.7%) at diagnosis and relapse. The PAS in 20 patients (66.7%) and 9 patients (30%) were positive at

Table 1. Characteristics of ALL patients.

Characteristics	ALL patients (n = 30)
Gender, n (%)	
Male	**17 (56.7%)**
Female	**13 (43.3%)**
Age, mean (range)	**6.7 (1 - 15 years)**
Immunophenotyping, n (%)	
B-ALL	**23 (76.7%)**
T-ALL	**7 (23.3%)**
Years of relapse, n (%)	
First-year	**8 (26.7%)**
Second-year	**11 (36.7%)**
Third-year	**11 (36.7%)**

ALL - acute lymphoblastic leukemia.

diagnosis and relapse, respectively. The comparison of the results of stains was significantly different between diagnosis and relapse ($p = 0.027$), so that the percentage of PAS-positive patients decreased at relapse. Comparison of staining results with immunophenotyping and hematological data in all patients showed that HLA-DR was significantly higher at diagnosis ($p = 0.011$) and CD10 expression was significantly higher at relapse ($p < 0.001$) in PAS-positive patients than negative ones. The expression of TdT ($p = 0.001$ at diagnosis, $p < 0.001$ at relapse), CD19 ($p = 0.002$ at diagnosis, $p = 0.006$ at relapse) CD5 ($p = 0.034$ at diagnosis, $p = 0.050$ at relapse), and CD7 ($p = 0.038$ at diagnosis, $p = 0.012$ at relapse) were significantly higher in PAS-positive patients than PAS-negative at both diagnose and relapse. Total WBC count and ALC were significantly higher in PAS-negative than PAS-positive patients at relapse ($p = 0.014$ and 0.043, respectively).

Correlation between immunophenotyping biomarkers and laboratory data at time of diagnosis and relapse

To evaluate the role of CD markers' expressions in blood cells, we analyzed the association between the expression of CD markers in B- and T-ALL cells with blood cell counts at diagnosis and relapse. There was a negative correlation between cMPO expression with Hb level ($r = -0.579$, $p = 0.049$), CD2 ($r = -0.409$, $p = 0.025$), CD3 ($r = -0.467$, $p = 0.009$), and CD4 expression ($r = -0.927$, $p = 0.003$) with blast percentage, and CD4 expression with Hb level ($r = -0.991$, $p < 0.001$) at diagnosis. Also, there was a positive correlation between CD3 expression and PLR ($r = 0.367$, $p = 0.046$) but a negative correlation between CD10 expression and LMR ($r = -0.363$, $p = 0.049$), ALC ($r = -0.365$, $p = 0.047$), and total WBC count ($r = -0.406$, $p = 0.026$). TdT expression had a negative correlation with platelet count ($r = -0.441$, $p = 0.017$), and so do CD2 expression with blast percentage ($r = -0.468$, $p = 0.009$), and CD19 expression with ALC ($r = -0.401$, $p = 0.028$) at relapse. On the other hand, by assessing the association between the expression of CD markers and blood cell counts with the age of patients at diagnosis and relapse, we found that there is a positive correlation between CD20 and TdT expression at diagnosis ($r = 0.480$, $p = 0.007$ and $r = 0.556$, $p = 0.002$) and relapse ($r = 0.443$, $p = 0.014$ and $r = 0.418$, $p = 0.024$) and the age of patients, respectively.

DISCUSSION

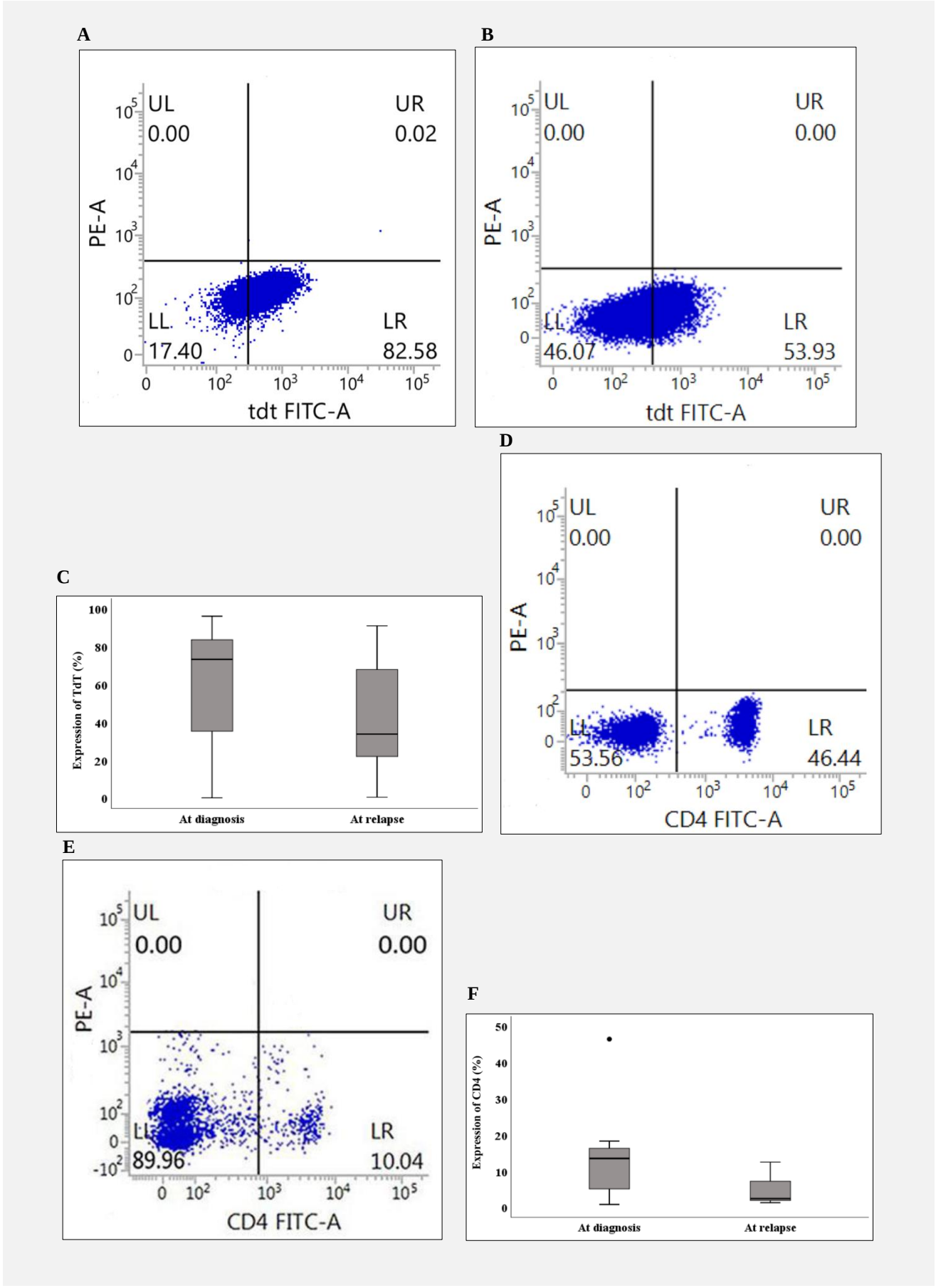
Diagnosis and monitoring of ALL is important in order to overcome this disease, for which the assessment of morphological and immunophenotypical features as main modality can be used [2,4]. Prognosis of ALL relapse depends on different factors such as time to relapse, the site of relapse, immunophenotype, and diagnostic features [1,12]. In this regard, we investigated the changes in the expressions of different CD markers and hematological findings both at diagnosis and relapse in children to explore the prognostic value of these markers in ALL.

It was revealed that the percentage of TdT expression was significantly higher at diagnosis compared with the time of relapse. These data concur with the results of Bayram et al. and Li et al., which reported that the TdT expression was lost in patients at relapse [7,13]. Although Kim et al. showed that TdT expression is associated with hematological relapse-free survival, overall survival, and good prognosis in ALL [14], we did not find a correlation between TdT expression and years of relapse. However, we found that TdT expression had a negative correlation with platelet count at relapse and a

Table 2. Immunophenotypic and hematological parameters changes of ALL patients at diagnosis and relapse.

Data	At the time of diagnosis Median (IQR) n	At the time of relapse Median (IQR) n	p-value
HLA-DR	83.15 (27.72 - 92.0) 30	79.70 (36.02 - 89.40) 30	0.517
IgM	3.10 (1.85 - 16.95) 13	4.10 (0.80 - 9.10) 5	0.180
TdT	73.30 (32.75 - 84.85) 29	33.80 (21.90 - 69.95) 29	0.004 **
CD2	5.75 (3.27 - 13.15) 30	8.25 (3.20 - 17.80) 30	0.469
CD3	5.90 (2.97 - 12.07) 30	8.60 (3.72 - 16.35) 30	0.168
CD4	13.50 (2.60 - 18.30) 7	3.50 (1.82 - 8.45) 12	0.043 *
CD5	6.05 (4.72 - 24.97) 30	13.60 (5.17 - 49.97) 30	0.258
CD7	7.20 (4.27 - 43.32) 30	12.50 (5.82 - 41.30) 30	0.318
CD8	6.70 (2.10 - 6.90) 7	5.05 (1.95 - 8.27) 12	0.499
CD4/CD8 ratio	1.58 (0.38 - 6.8) 7	1.30 (0.74 - 1.71) 12	0.310
CD10	74.80 (2.52 - 93.47) 30	47.80 (8.70 - 89.0) 30	0.299
CD13	2.50 (0.77 - 5.02) 30	2.95 (1.37 - 8.55) 30	0.376
CD19	87.60 (48.55 - 93.20) 30	81.10 (34.32 - 90.05) 30	0.344
CD20	9.70 (5.55 - 36.82) 30	10.10 (1.85 - 46.67) 30	0.636
CD33	3.85 (1.05 - 8.10) 30	2.75 (1.30 - 9.95) 30	0.517
Blast	87.50 (75.0 - 95.0) 30	76.50 (60.0 - 90.0) 30	0.064
WBC count (x 10^9 /L)	12.10 (7.50 - 29.52) 30	6.95 (4.30 - 18.67) 30	0.072
PLT (x 10^9 /L)	42.0 (17.25 - 82.25) 30	34.50 (18.0 - 83.50) 30	0.837
ANC (x 10^9 /L)	2.06 (1.06 - 6.08) 30	1.95 (0.64 - 4.20) 30	0.299
ALC (x 10^9 /L)	8.59 (5.07 - 18.11) 30	4.23 (2.15 - 11.28) 30	0.057
AMC (x 10^9 /L)	0.47 (0.13 - 1.42) 30	0.15 (0.09 - 0.54) 30	0.168
LMR (x 10^9 /L)	29.50 (11.13 - 66.35) 30	25.05 (10.25 - 72.85) 30	0.658
PLR (x 10^9 /L)	4.99 (1.0 - 9.02) 30	10.40 (3.65 - 28.50) 30	0.004 **
NLR (x 10^9 /L)	0.23 (0.14 - 0.39) 30	0.33 (0.12 - 1.26) 30	0.032 *
Data	**mean ± SD n**	**mean ± SD n**	**p-value**
cMPO	4.61 ± 4.8 12	2.85 ± 2.3 7	0.909
Hb (g/dL)	8.95 ± 2.3 30	9.25 ± 2.8 30	0.654

ALL - acute lymphoblastic leukemia, SD - standard deviation, IQR - interquartile range, cMPO - cytoplasmic myeloperoxidase, TdT - terminal deoxynucleotidyl transferase, HLA-DR - human leukocyte antigen-DR isotype, WBC - white blood cell, PLT - platelet, ANC – absolute neutrophil count, ALC - absolute lymphocyte count, AMC - absolute monocyte count, Hb - haemoglobin, LMR - lymphocyte/monocyte ratio, PLR - platelet/lymphocyte ratio, NLR - neutrophil/lymphocyte ratio.
*** - indicates statistical significance ($p < 0.05$). ** - indicates statistical significance ($p < 0.01$).**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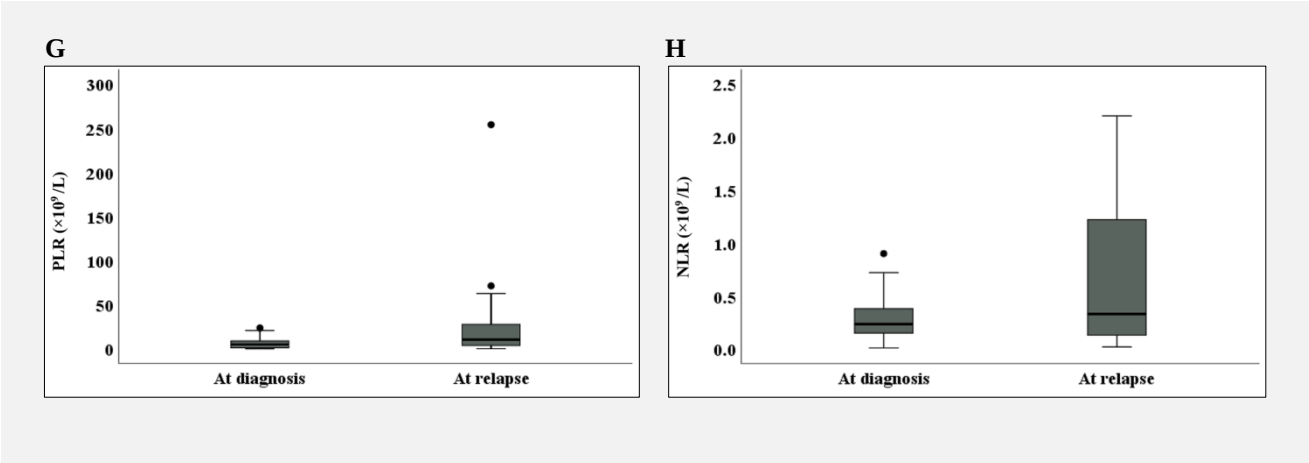

Figure 1. The different percentages of TdT, CD4 expressions, NLR and PLR in ALL patients.

(A) Representative dot plots of TdT expression in one ALL patient at diagnosis. (B) Representative dot plots of TdT expression in one ALL patient at relapse. (C) The percentage of TdT expression was higher at diagnosis than relapse in ALL patients (p = 0.004). (D) Representative dot plots of CD4 expression in one ALL patient at diagnosis. (E) Representative dot plots of CD4 expression in one ALL patient at relapse. (F) At the diagnosis of ALL, the percentage of CD4 expression was higher than relapse (p = 0.043). (G) The platelet/lymphocyte ratio (PLR) was lower at diagnosis than relapse (p = 0.004). (H) The neutrophil/lymphocyte ratio (NLR) was lower at diagnosis than relapse (p = 0.032). All p-values were calculated by the Wilcoxon signed-rank test.

positive correlation with the age of patients at both times. Lower platelet count and higher age of patients are poor prognostic factors in ALL [15,16]. It seems that a decrease of TdT expression at relapse correlates with higher platelet count and lower age of patients; so, a decline in this marker may have good prognostic value in ALL.

Also, we found that the percentage of CD4 expression was significantly higher at diagnosis than relapse. In contrast to our data, in a study by Li et al. it was reported that there was no significant difference in CD4 expression between diagnosis and relapse [13]. Mugairi et al. showed that CD4 expression in ALL patients was associated with complete remission rate, longer relapse-free survival, and overall survival [17]. In our study, a decrease of CD4 expression in ALL patients at relapse may be related to a higher risk of relapse [18]. We detected five patients with aberrant CD4 expression at relapse which may be related to poor prognosis in these patients. Moreover, Hussein et al. showed that T-cell antigen expression was associated with high-risk factors (age and/or WBC count), although it was not at a statistically significant level [18]. This differs from the findings presented here. We detected that there was a negative correlation between CD4 expression with blast percentage and Hb level at diagnosis.

On the other hand, Lustfeld et al. reported that higher CD4/CD8 ratios at diagnosis correlated with a favorable response to treatment [19]. Although in our study, the CD4/CD8 ratio was slightly higher at diagnosis than relapse with no significant difference, it can thus be suggested that a decrease in CD4/CD8 ratio at relapse may be associated with a higher risk of relapse.

In comparing blood counts at diagnosis with relapse, we found that NLR and PLR were significantly lower at diagnosis than relapse. Prior studies have noted the importance of high NLR association with poor overall survival in relapsed/refractory AML patients [20]. Higher NLR depends on an increase of ANC or decrease of ALC, which can act as effective factors in the risk of relapse. Furthermore, several reports have shown a direct and indirect relationship between ANC and ALC with the risk of relapse, respectively [21,22]. While our study did not show any correlation between NLR and risk of relapse, we observed that a decrease in ALC could be an effective factor in NLR and risk of relapse. Hence, it could conceivably be hypothesized that NLR can be associated with a higher risk of relapse in ALL patients. Another important finding was that we did not see any correlation between PLR and the risk of relapse in ALL patients. Bakouny et al. in a similar study reported that PLR was not related to the survival of CLL patients [23]. To evaluate the prognostic role of PLR in ALL patients, therefore, it appears that more investigation is needed. This experiment did not detect any significant difference between other immunophenotyping markers and hematological parameters at diagnosis and relapse. It seems possible that these results are due to statistical analysis, not biological reasons.

The results of this study indicated that there is a significant difference between cytochemistry staining at diagnosis and relapse, so that the percentage of PAS-positive patients decreased at relapse (20 of 29 ALL patients were PAS-positive at diagnosis). In a study by Mukda et al., it was shown that 70 out of 84 ALL patients were PAS-positive at diagnosis [24]. In another study, the

PAS staining was positive in all cases (31) of ALL [25]. Prior studies indicated that the PAS pattern was specific but less sensitive for diagnosis and subclassification of acute leukemias [24]. Thus, it would be better to use cytochemical staining combined with flow cytometry immunophenotyping for diagnosis of these patients. We also found that PAS had a significant, positive association with HLA-DR, TdT, and CD19 expression at diagnosis and CD10, TdT, and CD19 expression at relapse. Resende et al., in a study in this regard, found that PAS had a positive correlation with CD4, CD3, CD10, CD-19, and CD20 expression [25]. Furthermore, in our study PAS had a significant negative association with CD5 and CD7 expression in both times and total WBC count and ALC at relapse. This outcome is contrary to that of Deghady et al. who found that PAS had a positive association with CD5 expression [26]. Given the correlation between PAS stains with immunophenotyping markers, cytochemical stains can be used for the diagnosis of new ALL patients or patients with relapse. In regression analysis we determined that there was a negative correlation between cMPO expression with Hb level, CD2, and CD3 expression with blast percentage at diagnosis. The expression of myeloid antigens (CD-13, CD33, CD117, and cMPO) in ALL patients is related to poor prognosis [2]. Hence, high cMPO expression along with lower Hb levels, which is a poor prognostic factor in ALL patients [27], can be important factors for the risk of relapse. Also, there was a positive correlation between CD3 expression with PLR, but there was a negative correlation between CD10 expression and LMR, ALC, and total WBC count, CD2 expression and blast percentage, and CD19 expression and ALC at relapse. Another important finding was that similar to a study by Bayram et al., we did not find a correlation between immunophenotyping and time to relapse [7], but there was a positive significant association between ANC and years of relapse at diagnosis; so, the risk of relapse in the first year is higher in lower levels of these parameters. On the other hand, we did not find significant association between immunophenotyping and types of cells (B- and T-ALL) with years of relapse. Furthermore, in assessing the correlation of personal characteristics such as gender and age of patients with immunophenotyping and laboratory data, we found that males had significantly higher NLR at diagnosis and higher CD4 expression, total WBC count, and ANC at relapse than females. In the present study, like the Cézar et al. study, the ratio of male to female was higher, which is in agreement with the incidences of ALL worldwide [28].

Also, CD20 expression was higher at relapse than diagnosis, although the difference was not significant, and it had a positive correlation with the age of patients. Although older age is associated with a higher risk of relapse [1], prior studies have noted an association between CD20 expression and good prognosis in pediatric patients [28]. Therefore, given positive correlation between CD20 expression and the age of patients, it seems that further work is required to assess the prognostic role of CD20 expression in pediatric ALL.

The present study featured some limitations. First, our research was exclusively on pediatric patients, while it seems reasonable to investigate these relationships in adult patients. Third, the absence of cytogenetic findings and clinical characteristics of patients that could be applied to perform statistical analysis related to evaluating their prognostic role in the risk of relapse. Fourth, it would be useful to compare the percentage of PB blasts and BM blasts to assess their prognostic role.

It appears that some determinants, including TdT, CD4 expression, PLR, and NLR, could be applied as relapse prognostic factors in pediatric ALL patients. Furthermore, further work with larger sample sizes is warranted to fully understand the prognostic effects of immunophenotyping and laboratory findings in the risk of relapse in pediatric ALL patients.

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Authors Contributions:
M. B. conceived the manuscript and revised it. Z. G., M. Sh. and N. S. wrote the manuscript. M. F. and V. F. A. provided clinical data and information. M. Sh. and S. M. performed the technical tests.

Research Involving Human Participants and/or Animals:
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