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ORIGINAL ARTICLE

Absolute Lymphocyte Count (ALC) after Induction Treatment Predicts Survival of Pediatric Patients with Acute Lymphoblastic Leukemia

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Abstract Absolute Lymphocyte Count (ALC) has been recently established as a prognostic factor of survival in pediatric Acute Lymphoblastic Leukemia (ALL). A retrospective analysis of 132 patients treated according the BFM – ALLIC 2002 protocol was performed in a single institution. A possible association between ALC values and Overall Survival (OS) or Event-Free Survival (EFS) was evaluated at multiple time points during induction chemotherapy. ALC higher than 350 cells/μL measured on the 33th day of induction was associated with better Overall- and Event-Free Survival in both Kaplan-Meier (OS 88.6% vs. 40%; $p < 0.001$ / EFS 81.6% vs. 30%; $p < 0.001$) and Cox regression (OS HR 8.77 (3.31–23.28); $p < 0.001$) and EFS HR 6.61 (2.79–15.63); $p < 0.001$) analyses. There was no association between survival and measured ALC values from earlier time points (day of diagnosis, days 8 and 15) of induction therapy. Patients with low ALC values tend to have higher risk (MR or HR groups) and a higher age at diagnosis (>10 years). With help of day 33 ALC values of 350 cells/μL cutoff it was possible to refine day 33 flow cytometry (FC) Minimal Residual Disease (MRD) results within the negative cohort: higher ALC values were significantly associated with better survival. ALC on day 33 (350 cells/μL) remained prognostic for OS and EFS in multivariate analysis after adjusting it for age, cytogenetics, immunophenotype and FC MRD of induction day 33. According to these findings ALC on day 33 of induction is a strong predictor of survival in pediatric ALL.

Keywords ALC · All · Survival

Introduction

Acute lymphoblastic leukemia (ALL) is the most common hematological malignancy in childhood. Outcomes for the disease have significantly improved in last decades, mainly due to introduction of individualized therapeutic strategies based on risk stratification, and due to use of superior salvage regimes and better intensive care [1–3].

Multiple factors have been proven being predictive of outcome in pediatric ALL: initial white blood cell count (WBC), age, immunophenotype (B- or T-ALL), cytogenetics (t(9;22) or t(4;11)) and response to treatment (prednisone response on day 8 and blast reduction in bone marrow on day 15 and 33) are used in current BFM protocols to determine potential hazard for outcome [4–6]. Minimal Residual Disease (MRD) identified by flow cytometry or molecular genetic techniques is the most reliable independent predictor of survival and relapse in pediatric ALL [4, 5]. Reported overall survival (OS) of childhood ALL is 80–90% with current therapy protocols, and it might exceed 95% in low-risk patients [3].

A further improvement of therapy outcome in pediatric ALL might be achieved by using more accurate risk stratification strategies. To assess individual hazard better, identification of novel prognostic factors is useful.

Recent studies established Absolute Lymphocyte Count (ALC) as a prognostic factor of outcome in various hematological malignancies and solid tumors. Higher ALC measured during induction chemotherapy was associated with superior survival in both adult and pediatric ALL, pediatric acute

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myeloblastic leukemia (AML), adult follicular lymphomas, and Ewing sarcoma [7–18]. Additionally, ALC was found to be predictive of outcome after hematopoietic stem cell transplantation (HSCT) in adults with AML, Hodgkin- and Non-Hodgkin lymphomas, myeloma multiplex, breast cancer and after HSCT in pediatric Acute Myeloblastic Leukemia (AML) and ALL [19–22]. The observation, that early lymphocyte recovery is associated with better outcome, reinforced the idea that lymphocytes, especially NK- and T-cell-subsets constitute an effective natural mechanism of the immune system against tumor cells [23, 24]. ALC was found to be prognostic of overall survival and event-free survival (EFS) in multiple studies [7, 9, 11–15, 17, 18]. However, no universal ALC measurement time point and cut-off value could be determined so far. In previous reports various measurement time points (day 13, day 28, day 29, day 36 and day 43) and ALC cut-off values (350 cells, 500 cells, 900 cells, 1000 cells and 1500 cells) were found to be predictive of outcome [7, 9, 11–15, 17, 18]. Additionally, the ratio ALC-22/ALC-0 as of ALC on day-22 (ALC-22) and ALC on day of diagnosis (ALC-0) with <10% cut-off was introduced as an independent risk factor for survival [9]. It was expected to serve as a universal ALC marker, which might be evenly applicable in different therapeutic protocols.

Recently, day 29 ALC (ALC-29) with 350 cells/μL cut-off value was found to be associated with OS in patients treated according the BFM - ALLIC 2009 protocol, which uses the same therapy regimen during the first 33 days of induction, as our current study [12]. It is interesting to investigate, whether we can detect similar prognostic markers in patients receiving similar therapies.

Our study aimed to determine the predictive value of ALC and ALC ratios for OS and EFS in a group of 132 pediatric patients treated according the BFM -ALLIC 2002 therapeutic protocol. Potentially prognostic different ALC cut-off values and time points were evaluated in different subgroups of patients.

Methods

Patients and Treatment

132 pediatric patients (age ≤ 18 years) with newly diagnosed ALL from December 2002 to June 2011 at the 2nd Children's Hospital of Semmelweis University Budapest were enrolled in the study. All patients were treated according the BFM - ALLIC 2002 protocol. Risk stratification and treatment modalities have been published elsewhere [6]. The first 33 days of induction consisted of a monotherapy with increasing doses of prednisone between days 1–7, followed by a treatment with a combination of 5 drugs (prednisone, vincristine, daunorubicin, L-asparaginase and intrathecal methotrexate).

Analysis and Statistical Methods

We reviewed patient's medical charts to determine information with clinical importance, including sex, age at diagnosis, risk group, central nervous system (CNS) status, initial WBC, peripheral blast count on day 8, bone marrow (BM) morphology with blast percentage at diagnosis and on days 15 and 33, cytogenetics changes. Although MRD stratification was not utilized in the BFM - ALLIC 2002 protocol, analysis of residual leukemia cells in BM sample were performed by using flow cytometry on days 15 and 33 (FC-MRD-15 and FC-MRD-33) and quantitative PCR on day 33 (PCR-MRD-33) as part of a pilot study. FC results below 10% on day 15 and 0.1% on day 33 of induction were considered as favorable prognostic markers of survival.

ALC was determined at time of diagnosis (ALC-0) and on days 8, 15 and 33 (ALC-8, ALC-15 and ALC-33). In following, ratios of ALC values (ALC-8/ALC-0, ALC-15/ALC-0 and ALC-33/ALC-0) were calculated. Additional flow cytometry immunophenotyping and quantitative PCR were used to determine MRD status of patients.

The Kaplan-Meier estimate was used to determine OS and EFS, and the log-rank test was utilized to compare the survival distributions of patient groups. OS was defined as time from diagnosis to death from any cause or to time point of the last follow up examination. EFS was defined as time from diagnosis to any kind of treatment failure (death, relapse or secondary malignancy) or in case of no event to time of the last follow up examination. Additionally, Cox's proportional hazards model was used to test variables in univariate and multivariate analysis. Hazard ratios were calculated with 95% confidence interval. Standard descriptive statistics, as mean or median and range, or percentage of cases were determined. Subsequently, continuous variables between smaller patient groups were compared with the Mann-Whitney U-test or with the independent samples t-test, while categorical variables were analyzed with chi-square test.

ALC values at multiple time points of induction were tested as continuous and discrete variables. ALC cut-off values, which have been prognostic for survival in previous occasions (350−/500−/900−/1000−/1500 cells/μl), were evaluated [7, 9, 11–15, 17, 18]. For ALC-ratios we used 10% as cut-off value, which has been associated with survival previously [9].

Obtained data was analyzed by the SPSS 20.0 program.

Results

Patient Characteristics

Main characteristics and survival of patients are summarized in Table 1. Our pediatric cohort had 5-year OS 85.6% and EFS 78.8%. The sex ratio was unequal within the cohort, as 47

Table 1 Demographics and survival according to evaluated variables

Variable	n (%)	OS (%)	*p*-value	EFS (%)	*p*-value
Sex					
male	85 (64%)	84.7	0.718	74.1	0.104
female	47 (36%)	87.2		87.2	
Age at diagnosis					
< 6 years	76 (58%)	93.4	0.003	86.8	0.009
≥ 6 years	56 (42%)	75		67.9	
< 10 years	99 (75%)	90.9	0.002	84.8	0.002
≥ 10 years	33 (25%)	69.7		60.6	
Phenotype					
pre B	108 (82%)	89.8	0.007	83.3	0.010
pre T	23 (17%)	65.2		56.5	
biphenotypic	1 (0.8%)	100		100	
Initial WBC					
< 20G/L	86 (65%)	88.4	0.199	82.6	0.131
≥ 20G/L	46 (35%)	80.4		71.7	
< 50G/L	109 (83%)	89	0.009	81.7	0.057
≥ 50G/L	23 (17%)	69.6		65.2	
Cytogenetics					
favourable	124 (95%)	87.1	0.005	80.6	0.001
unfavourable	6 (5%)	50		33.3	
Prednisone response					
good	113 (87%)	86.7	0.500	80.5	0.265
bad	17 (13%)	82.4		70.6	
Risk group					
SR	45 (34%)	95.6	0.002	88.9	<0.001
IR	60 (45.5%)	86.7		85	
HR	27 (20.5%)	66.7		48.1	
ALC-33					
< 1000 cells/μl	44 (35%)	77.3	0.070	70.5	0.129
≥ 1000 cells/μl	80 (65%)	88.8		81.2	
< 500 cells/μl	18 (15%)	61.1	0.001	44.4	<0.001
≥ 500 cells/μl	106 (85%)	88.7		83	
< 350 cells/μl	10 (8%)	40	<0.001	30	<0.001
≥ 350 cells/μl	114 (92%)	88.6		81.6	
ALC-8/ALC-0					
< 10%	16 (18%)	75	0.101	75	0.340
≥ 10%	72 (82%)	90.3		84.7	
ALC-15/ALC-0					
< 10%	24 (28%)	87.5	0.838	87.5	0.604
≥ 10%	62 (72%)	88.7		82.3	
ALC-33/ALC-0					
< 10%	20 (24%)	70	0.009	65	0.022
≥ 10%	63 (76%)	92.1		87.3	

females (36%) and 85 males (64%) were diagnosed with ALL in the clinic during the reviewed time period. Median age at diagnosis was 4.9 years (range: 1.1–16.9 years). At time of diagnosis 76 children (58%) were younger than 6 years old and 99 (75%) patients were younger than 10 years old. In majority of cases, the B-cell lineage was affected (n = 108.8%), while a smaller group of patients had T-cell ALL (n = 23.2%). One patient presented with biphenotypic ALL (0.8%). Median WBC at diagnosis was 9.22×10^3 cells/μL (range 0.7–710.36×10^3 cells/μL). Median ALC was 4.33×10^3 cells/μL (0–617.70×10^3 cells/μL) at diagnosis, 1.24×10^3 cells/μL (0–122×10^3 cells/μL) on day 8, 0.9×10^3 cells/μL (0–11.2×10^3 cells/μL) on day 15 and 1.37 (0–15.3×10^3 cells/μL) on day 33 of induction.

All together, 19 patients died due to disease progression or relapse. In total 22 relapses occurred after complete remission.

Prognostic Value of Known Factors

Initially, predictive value of known risk factors were tested (Table 1). Age at diagnosis 1–6 years, cytogenetics (no t(9;22) or t(4;11)), B-ALL phenotype and favorable BM morphology on days 15 and 33 were associated with superior OS and EFS. Other previously established factors, as absolute blast count on day 8 of induction or initial WBC with 20×10^3 cells/μL cut-off value were not predicting for survival. In contrast, when the higher cut-off value 50×10^3 cells/μL was used, which is adopted in other protocols, initial WBC was significantly associated with OS and EFS.

BM morphology and FC-MRD risk stratification on days 15 and 33 of induction were excellent predictors of outcome (Table 2). PCR-MRD-15 results were available in only small number of cases, which limits the interpretation of this data. PCR-MRD-33 results were significantly associated with OS and EFS.

The Cox regression analysis revealed similar associations between known markers and survival, as the log-rank analysis (Table 3).

Predictive Value of ALC

ALC at diagnosis and on days 8 and 15 were not prognostic for survival with neither of tested cut-off values (Supplemental Table 1). ALC-33 with a cut-off value 500 cells/μL was significantly associated with OS and EFS (Table 1). Particularly, patients with ≥500 cells/μL had excellent OS (88.7% vs. 61.1%; p = 0.001) and EFS (83% vs. 44.4%; $p < 0.001$). When the cut-off value 350 cells/μL was applied, we detected even larger difference in OS (88.6% vs. 40%; $p < 0.001$) and EFS (81.6% vs. 30%; $p < 0.001$) between patient groups (Fig. 1a). There was no significant association between higher cut-off values (ALC 1000 cells/μL and 1500 cells) and survival of patients (Supplemental Table 1.)

Low lymphocyte count might present in context of sepsis, which often accompanies induction chemotherapy [29]. In total, 4 patients died during induction therapy and 3 of them presented with ALC values below 500 cells/μL. As it was not possible to

Table 2 MRD risk stratification

Variable	n (%)	OS (%)	*p*-value	EFS (%)	*p*-value
BM morphology day 15					
M1	49 (59%)	89.3	<0.001	88	<0.001
M2	26 (31%)				
M3	8 (10%)	50		25	
FC-MRD day 15					
< 0.1%	48 (37%)	89.1	0.017	83.6	0.003
0.1–9.9%	62 (48%)				
≥ 10%	20 (15%)	70		55	
PCR-MRD day 15					
neg	19 (73%)	94.7	0.425	94.7	0.082
pos	7 (27%)	85.7		71.4	
BM morphology day 33					
M1	97 (96%)	89.7	<0.001	84.5	<0.001
M2	2 (2%)	25		0	
M3	2 (2%)				
FC-MRD day 33					
< 0.1%	99 (77%)	92.9	0.002	86.7	0.003
≥ 0.1%	29 (23%)	73.3		63.3	
PCR-MRD day 33					
neg	76 (87%)	93.4	<0.001	86.8	<0.001
pos	11 (13%)	63.6		45.5	

determine, whether sepsis resulted in low lymphocyte counts or not, we decided to perform survival analysis excluding all patients who died during induction. Patients with ALC-33 ≥ 500 cells/μL had better OS (89.4% vs. 73.3%; $p = 0.059$) and EFS (83.7% vs. 53.3%; $p = 0.004$), but *p*-value for OS was slightly above the criterion for significance. Subsequently, the cut-off value 350 cells/μL was tested in the reduced study cohort and it was found to be associated with both OS (89.3% vs. 57.1%; $p = 0.005$) and EFS (82.1% vs. 42.9%; $p = 0.005$).

Finally, we performed a Cox analysis testing ALC values with various cut-off values from different time points (Table 3). ALC-33 with cut-off value 350 cells/μL was significantly associated with OS (HR 8.77; $p < 0.001$) and EFS (HR 6.61; $p < 0.001$), even when patients who died during induction were excluded from analysis: OS (HR 5.17; $p = 0.011$) and EFS (HR 4.2; $p = 0.009$). However, the cut-off value 500 cells/μL was only significantly associated with EFS in the reduced patient cohort (HR 3.37; $p = 0.007$), but not with OD (HR 2.86; $P = 0.072$). Higher ALC cut-off values (1000 cells/μL and 1500 cells) were not associated with better survival (Table 3).

ALC values tested as a continuous variables were not prognostic of OS or EFS in neither of analyzed time points (Table 3).

Finally, ALC-33 with 350 cells/μL cut-off remained prognostic for OS and EFS in multivariate analysis after adjusting it for age, cytogenetics, immunophenotype and FC-MRD-33 (Table 4).

Predictive Value of ALC Ratios

Multiple ALC ratios (ALC-8/ALC-0, ALC-15/ALC-0 and ALC-33/ALC-0) were tested with 10% cut-off value (Tables 1 and 3). According to our results, the ALC-33/ALC-0 ratio was predictive of survival when using the Kaplan-Meier estimate or Cox's proportional hazards model. Patients with results over 10% presented with excellent OS (92.1% vs. 70%; $p = 0.009$) and EFS (87.3% vs. 65%; $p = 0.022$) while results below 10% were associated with inferior outcome. Other tested ALC ratios had no prognostic significance.

The ALC-33/ALC-0 ratio with 10% cut-off value remained a strong predictor of OS and EFS in univariate Cox regression (Table 3). However, the ratio was not significantly associated with OS and EFS, when tested in a multivariate model after adjusting it for age, cytogenetics, immunophenotype and FC-MRD-33 (Table 4).

Patient Characteristics According to ALC-33

In following, the study cohort was divided into two groups according ALC-33 values (<350 cells/μL and ≥350 cells/μL) to evaluate whether these findings are more likely to be found with specific clinical features (Table 5). Patients with <350 cells/μL ALC-33 were more likely to be older than 6 years, and the difference was even more explicit when patients were divided in subgroups according age below or over 10 years. Median age at diagnosis was 11.39 years among patients with ALC-33 < 350 cells/μL, compared to 4.57 years among patients with ≥350 cells/μL. Furthermore, age analyzed as a continuous variable in was significantly associated with ALC-33 with 350 cells/μL cut-off ($p < 0.001$).

Additionally, patients with <350 cells/μL tend to have higher than standard risk (SR) after risk stratification, as none of them presented with SR.

In following, we calculated OS and EFS within patient subgroups according to ALC-33 with 350 cells/μL cut-off. ALC-33 with ≥350 cells/μL was significantly associated with OS (33.3% vs. 92%, $p < 0.001$) and EFS (33.3% vs. 90%, $p < 0.001$) in the group of patients with intermedier risk (IR) (Fig. 1b). However, when we excluded patients who died during induction, the sample size was too small for further survival analysis.

ALC-33 with ≥350 cells/μL was significantly associated with OS (28.6% vs. 79.2%; $p = 0.002$) and EFS (14.3% vs. 70.8%; $p = 0.001$) in patients older than 10 years, even when patients with early death during induction were excluded (OS 40% vs. 82.6%; 0.022; EFS 20% vs. 72.9%; $p = 0.007$).

MRD Risk Stratification

Subsequently we calculated survival within groups of FC-MRD-33 negative and positive patients according to ALC. Low ALC (<350 cells/μL) was associated with inferior OS (66.7% vs. 94%;

Table 3 Univariate cox regression analysis

Variable	Overall survival		Event-free survival	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Age at diagnosis (<1 or ≥6 years)	4.13 (1.49–11.47)	0.007	2.70 (1.24–5.84)	0.012
Age at diagnosis (<1 or ≥10 years)	3.84 (1.56–9.48)	0.003	3.11 (1.48–6.54)	0.003
Sex	0.84 (0.32–2.20)	0.718	0.48 (0.20–1.19)	0.111
Initial WBC ($20 \times 10^3/\mu L$)	0.21 (0.73–4.40)	0.206	1.76 (0.84–3.70)	0.136
Initial WBC ($50 \times 10^3/\mu L$)	3.24 (1.28–8.25)	0.014	2.18 (0.96–4.95)	0.063
Cytogenetics t(9;22) or t(4;11)	4.96 (1.44–17.10)	0.011	5.30 (1.82–15.42)	0.002
Prednisone response	1.53 (0.44–5.28)	0.504	1.73 (0.65–4.56)	0.271
Risk group	2.95 (1.51–5.76)	0.002	2.81 (1.63–4.84)	<0.001
Immunophenotype	2.76 (1.27–5.96)	0.010	2.29 (1.17–4.50)	0.016
FC-MRD day 15	1.76 (1.08–2.88)	0.023	1.79 (1.20–2.67)	0.004
FC-MRD day 33	4.49 (1.63–12.39)	0.004	3.40 (1.52–7.59)	0.003
PCR-MRD day 15	2.93 (0.18–46.97)	0.447	6.41 (0.58–71.08)	0.130
PCR-MRD day 33	8.01 (2.12–30.20)	0.002	7.48 (2.67–20.95)	<0.001
BM morphology day 15	2.66 (1.45–4.87)	0.002	3.00 (1.78–5.05)	<0.001
BM morphology day 33	4.10 (2.01–8.03)	<0.001	4.57 (2.47–8.44)	<0.001
ALC-0 (continuous)	1.01 (0.99–1.01)	0.743	1.00 (0.99–1.01)	0.956
ALC-8 (continuous)	0.99 (0.94–1.04)	0.613	0.99 (0.95–1.03)	0.542
ALC-15 (continuous)	1.05 (0.77–1.43)	0.783	0.95 (0.67–1.34)	0.750
ALC-33 (continuous)	1.09 (0.90–1.32)	0.365	1.15 (0.99–1.33)	0.060
ALC-33 (<350 cells/μl)	8.77 (3.31–23.28)	<0.001	6.61 (2.79–15.63)	<0.001
ALC-33 (<350 cells/μl) w/o induction deaths	5.17 (1.45–18.40)	0.011	4.20 (1.43–12.33)	0.009
ALC-33 (<500 cells/μl)	4.45 (1.75–11.34)	0.002	4.42 (2.04–.9.61)	<0.001
ALC-33 (<500 cells/μl) w/o induction deaths	2.86 (0.91–9.03)	0.072	3.37 (1.39–8.12)	0.007
ALC-33 (<1000 cells/μl)	2.25 (0.91–5.54)	0.078	1.76 (0.84–3.71)	0.135
ALC-33 (<1500 cells/μl)	1.14 (0.46–2.84)	0.777	1.31 (0.61–2.80)	0.483
ALC-8/ALC-0 (<10%)	2.69 (0.79–9.12)	0.115	1.73 (0.55–5.45)	0.346
ALC-15/ALC-0 (<10%)	1.15 (0.29–4.45)	0.838	071 (0.20–2.56)	0.606
ALC-33/ALC-0 (<10%)	4.25 (1.30–13.93)	0.017	3.09 (1.12–8.52)	0.030
ALC-33/ALC-0 (<10%) w/o induction deaths	2.84 (0.76–10.57)	0.120	2.19 (0.72–6.71)	0.168

$p = 0.009$) and EFS (50% vs. 88.1%; $p = 0.005$) in FC-MRD-33 negative patients, but not in FC-MRD positive patients (Fig. 1c). When using higher ALC cutoff values, we found no significant difference in survival of patient groups according to FC-MRD-33. There was no association within PCR-MRD-33 negative and positive groups and ALC-33 values.

Discussion

We analyzed the association of prognostic factors with outcome in a study group of 132 pediatric ALL patients treated according the BFM - ALLIC 2002 protocol. This allowed the confirmation of predictive value of known markers for survival: age 1–6 years, favorable cytogenetics (no t(9;22) or t(4;11)), B-ALL phenotype, BM morphology on days 15 and 33 and negative MRD-status on day 15 by FC and on day 33 by FC or PCR were associated with superior outcome in examined patients. Interestingly, initial WBC with 20×10^3 cells /μL cut-off value and peripheral blast count on day 8 failed to show prognostic effect for OS and EFS. However, when 50×10^3 cells/μL cut-off value was selected, which is used in other protocols, WBC was a significant predictor of outcome. Initial WBC and prednisone response are factors used for risk stratification during induction. Patients with high WBC at diagnosis or higher blast count on day8 receive augmented chemotherapy, which contributes to improved outcome, but most likely also results in less significant association of these markers with long-term survival of patients.

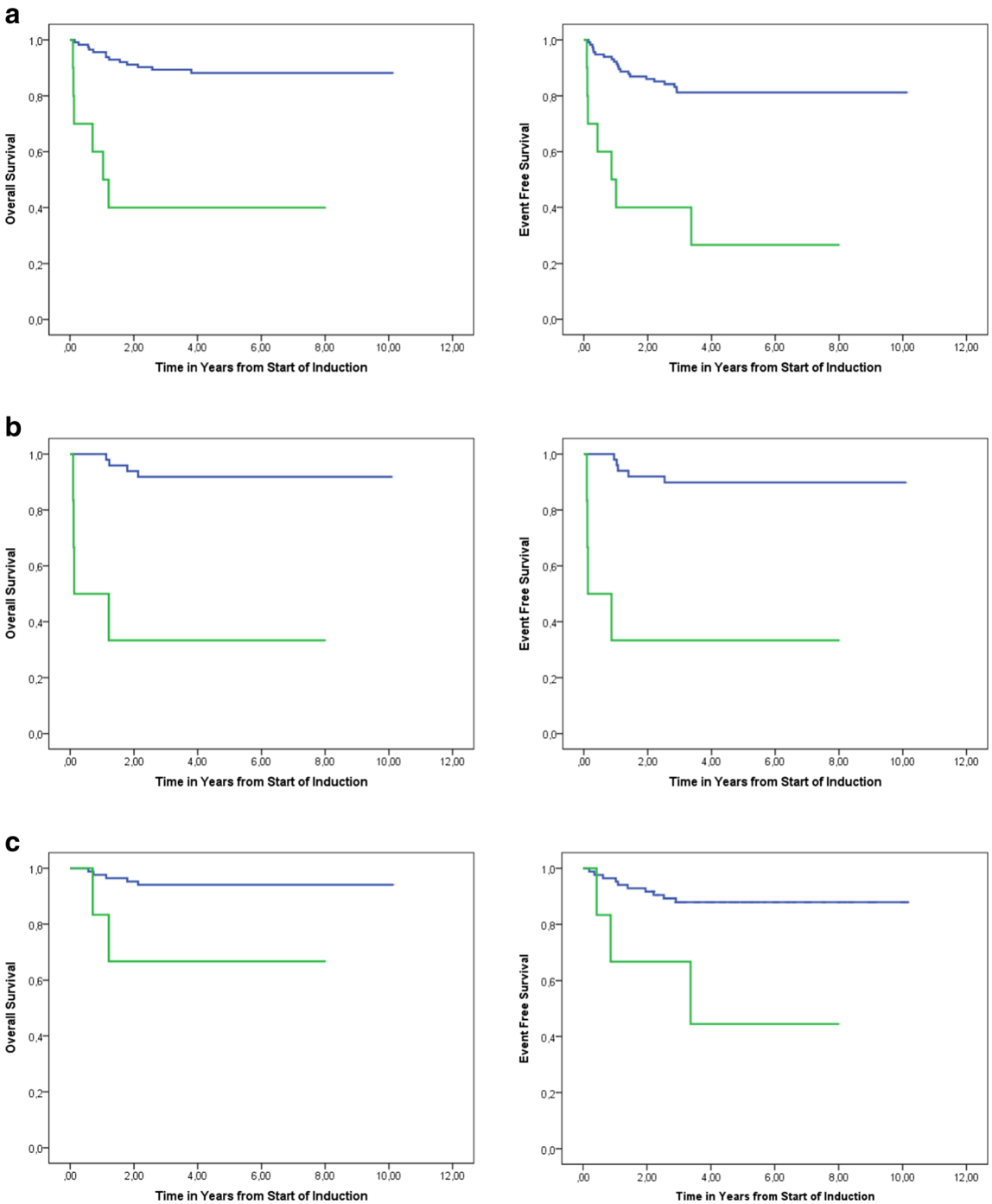

Fig. 1 OS and EFS in different patient subgroups according to ALC-33 with 350 cells/μl cut-off value. Blue lines indicate results from patients with higher ALC vales. **a**: OS and EFS within the group of 132 pediatric patients with ALL according ALC-33 with 350 cells/μl cut-off value. OS 40% vs. 88.6%, $p < 0.001$ and EFS 30% vs. 81.6%, $p < 0.001$. **b**: OS and EFS within the group of patients with IR risk group according ALC-33 with 350 cells/μl cut-off value. OS 33.3% vs. 92%, $p < 0.001$ and EFS 33.3% vs. 90%, $p < 0.001$. **c**: OS and EFS within the group of flow cytometry MRD-33 negative patients according ALC-33 with 350 cells/μl cut-off value. OS 66.7% vs. 94%; $p = 0.009$ and EFS 50% vs. 88.1%; $p = 0.005$

Recent studies have shown, that ALC during induction is associated with outcome in pediatric and adult ALL patients [7, 9, 11–15, 17, 18]. So far, different prognostic ALC cut-off values and measurement time points have been identified, most likely due to marked differences between patients' characteristics and therapy protocols.

We evaluated ALC on day of diagnosis and on days 8, 15 and 33 of induction, which are important decision-making time point in risk stratification according the BFM - ALLIC

Table 4 Multivaraite Cox regression analysis

Variable	Overall survival		Event-free survival	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Age at diagnosis (<1 or ≥6 years)	2.91 (0.83–10.17)	0.094	1.76 (0.72–4.29)	0.215
Cytogenetics t(9;22) or t(4;11)	8.44 (2.13–33.40)	0.002	5.76 (1.86–17.89)	0.002
Immunophenotype	2.40 (0.87–6.65)	0.091	1.76 (0.74–4.19)	0.200
FC-MRD day 33	3.11 (0.99–9.73)	0.051	2.34 (0.94–5.81)	0.067
ALC-33 (≥350 cells/μl)	6.29 (1.52–25.92)	0.011	4.62 (1.42–15.07)	0.011
Age at diagnosis (<1 or ≥6 years)	6.51 (0.69–60.96)	0.101	2.04 (0.52–7.95)	0.305
Cytogenetics t(9;22) or t(4;11)	33.22 (1.46–754.33)	0.028	58.88 (2.72–1275.04)	0.009
Immunophenotype	20.9 (0.63–6.96)	0.230	1.85 (0.61–5.59)	0.277
FC-MRD day 33	2.53 (0.56–11.47)	0.228	1.48 (0.41–5.30)	0.550
ALC-33/ALC-0 (<10%)	1.40 (0.29–6.85)	0.678	1.78 (0.49–6.40)	0.380

2002 protocol. The selected time point for ALC measurement on day 33 allowed a further refinement of FC-MRD-33 results. We found no link between ALC values from early time points (ALC-0, ALC-8 and ALC-15) and outcome of disease.

Table 5 Patient characteristics according to ALC-33

Variable	ALC-33 <350 cells/μL (*n* = 10)	ALC-33 ≥350 cells/μL (*n* = 114)	*p*-value
Sex			
male	7 (70%)	75 (66%)	0.787
female	3 (30%)	39 (34%)	
Age at diagnosis			
< 6 years	1 (10%)	70 (61%)	0.002
≥ 6 years	9 (90%)	44 (39%)	
< 10 years	3 (30%)	90 (79%)	0.001
≥ 10 years	7 (70%)	24 (21%)	
Phenotype			
pre B	8 (80%)	94 (82%)	0.926
pre T	2 (20%)	19 (17%)	
biphenotypic	0 (0%)	1 (1%)	
Initial WBC			
< 20G/L	8 (80%)	72 (63%)	0.286
≥ 20G/L	2 (20%)	42 (37%)	
< 50G/L	8 (80%)	95 (83%)	0.788
≥ 50G/L	2 (20%)	19 (17%)	
Cytogenetics			
favourable	10 (100%)	106 (93%)	0.453
unfavourable	0 (0%)	6 (7%)	
Prednisone response			
good	8 (80%)	98 (87.5%)	0.501
bad	2 (20%)	14 (12.5%)	
Risk group			
SR	0 (0%)	42 (37%)	0.048
IR	6 (60%)	50 (44%)	
HR	4 (40%)	22 (19%)	

However, ALC-33 with 350 cells/μL cut-off was significantly associated with OS and EFS in pediatric ALL. A cut-off value of 500 cells/μL was found to be prognostic for survival in the entire study cohort, but it lost its significance for predicting OS, when patients with induction deaths were excluded from the group. Therefore we decided to utilize 350 cells/μL cut-off for ALC-33. In a recent report ALC on day 29 was associated with OS [12]. In this study, 350 cells/μL cut-off value was found to be prognostic for outcome in this group of pediatric patients, who were treated with identical induction therapy to our cohort.

According to our findings, low ALC-33 was more prevalent among patients older than 10 years. By using ALC-33 as a marker for these patients, we were able to identify a subgroup with extremely poor outcome (OS 79.2% vs. 28.6%; $p = 0.002$ and EFS 70.8% vs. 14.3%; $p = 0.001$). In a previous report about 198 adult ALL patients (median age at diagnosis 38 years) ALC-28 with 350/μL cut-off value was significantly associated with length of OS and EFS [17]. Additionally, an initial study by De Angulo described an association between ALC-28 with 350/μL cut-off and relapse-free survival (RFS) in a pediatric group with high median age (11 years) [11]. It is known, that specific cytogenetic markers are associated with older age in pediatric patients with ALL. It might be speculated, that low ALC during induction can be linked to a genetic defect.

At time, molecular genetic- or flow cytometry MRD stratification during- and at the end of induction are the most reliable methods to predict outcome in children with ALL. However, relapse might occur in patients with negative FC-MRD-33 results as well. According to Rabin et al. ALC-29 was applicable to refine MRD-based risk stratification [13]. In their report, ALC-29 ≥ 1500 cells was associated with superior outcome in both MRD-29 negative and positive patient subgroups.

We analyzed ALC-33 with 350 cells/μL cut off in association with MRD-33 results. ALC results were prognostic for OS and EFS within patients with negative FC-MRD-33

results. We found no association of ALC-33 with outcome in FC-MRD positive- and none of PCR-MRD results. ALC-33 is a promising marker for predict relapse in MRD negative patients, in patients with usually favorable prognosis.

Recently, Cheng et al. proposed ALC-22/ALC-0 ratio with 10% cut-off as a novel prognostic marker for outcome in pediatric ALL [9]. According to their findings, this ALC ratio had more predictive value compared to single ALC values during induction. We tested 3 different ratios with available data (ALC-8/ALC-0, ALC-15/ALC-0 and ALC-33/ALC-0) and we identified ALC-33/ALC-0 with 10% cut-off to be associated with OS and EFS. However, in contrast to ALC-33, the ALC-33/ALC-0 ratio with 10% cut-off did not reach statistical significance neither in a patient group, when induction deaths were excluded, nor in multivariate Cox analysis.

Absolute Lymphocyte Count (ALC) is a simple, inexpensive and easily accessible marker even in resource-poor countries where no high cost equipment and examinations are available. In our retrospective analysis of pediatric ALL ALC-33 was found to be an independent marker with high prognostic value for outcome.

In summary, ALC-33 could be utilized to identify patients with favorable outcome (>350 cells/μl) and patients with dismal prognosis (<350 cells/μl), with survival rates similar to the HR group. ALC is especially suitable to predict OS and EFS in patients older than 10 years.

Strikingly, ALC-33 was a good marker of outcome in patients who tested negative in FC-MRD-33. With inclusion of ALC in risk stratification, future protocols might identify patients with poor prognosis, who cannot be detected with recently applied markers. Patients with dismal prognosis might receive augmented chemotherapy, while side-effects of therapy can be reduced in patients with good prognosis. Our findings have to be confirmed in future clinical studies in larger patient groups.

ALC, Absolute lymphocyte count; ALL, Acute lymphoblastic lekemia, AML, Acute myeloblastic leukemia; BFM, Berlin-frankfurt-münster; BM, Bone marrow; EFS, Event-free survival; FC, Flow citometry; HSCT, Hematopoietic stem cell transplantation; MRD, Minimal residual disease; OS, Overall survival; PCR, Polymerase chain reaction.

Compliance with Ethical Standards

Conflict of Interest The authors declare no competing financial interests.

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