

# Evaluation of sample quality as preanalytical error in flow cytometry analysis in childhood acute lymphoblastic leukemia

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## ARTICLE INFO

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## ABSTRACT

### **Introduction**

Acute lymphoblastic leukemia (ALL) is the most common cancer among children. The intensity of chemotherapy and further therapeutic decisions depend on several prognostic factors, including response to initial treatment by examining peripheral blood (PB), bone marrow (BM) and cerebrospinal fluid (CSF) samples at certain time points. (e.g. day 15 BM). Sample quality is crucial for the correct risk assessment.

### **Patients and methods**

We aimed to explore the rate of inadequate samples as a source of preanalytical error. We retrospectively analyzed flow cytometry results of BM (day 15 and day 33) and CSF samples from children with ALL in different cohorts focusing on PB contamination and viable cell ratio among nucleated cells. We also compared viable cell percentages in native and stabilized CSF samples.

## **Results**

Due to PB contamination (erythroid precursors < 2%) 12.5% of day 15 and 14% of day 33 BM samples were inadequate for flow cytometry risk stratification. Significantly fewer CSF samples had to be considered inadequate for analysis (defined as viable cells < 30%) in the subgroup of stabilized samples compared to native samples. Four of the CSF samples from children with ALL had identifiable malignant cell population despite the low viable cell percentage.

## **Discussion**

Poor sample quality can hamper risk stratification and further therapeutic decision in childhood ALL. Despite low viable cell count malignant cell populations may still be identified in a CSF sample, therefore establishing a certain cut-off point for viable cells is difficult.



## **INTRODUCTION**

Acute lymphoblastic leukemia (ALL) is the most common cancer among children still responsible for most deaths from cancer under 20 years of age, despite the tremendous improvement of survival since 1948, when Farber et al. successfully induced remission in children with ALL by administering aminopterin (1,2). ALL can be either B-cell or T-cell type with 85% of the cases being B-cell precursor ALL (BCP-ALL) and 15% T-ALL. The core of modern-day treatment of ALL is still the administration of combined chemotherapy developed by Riehm et al. in the 1970s (3). The intensity of chemotherapy and further therapeutic decisions depend on several prognostic factors including the clinical features of the patients on presentation, the genetic features of the leukemic cells, central nervous system (CNS) status and response to initial treatment (1). Sample quality is crucial in the correct risk assessment. A common preanalytical error

can be the contamination of either the bone marrow (BM) or the cerebrospinal fluid (CSF) sample with peripheral blood (PB). The sample must also contain enough viable cells for analysis, carried out either by morphological or by flow cytometric studies (4). According to the AIEOP BFM 2009 protocol evaluation of minimal residual disease (MRD) in the PB on day 8 and in the BM on day 15 and day 33 is important for adjusting treatment intensity (4). The possibility of diluting the BM specimen with PB should be minimized as a diluted sample may result in underestimating residual blast percentage and wrong risk assessment. According to the ALL IC-BFM 2009 Flow MRD SOP a day 15 BM cannot be used for risk stratification if the percentage of the erythroid precursors is below 2% (5), as PB contamination in this case is highly suspected. However, those inadequate samples containing more than 10% pathological blasts can still be reported and used for risk stratification as a Flow High Risk case.

CNS involvement needs administration of intrathecal chemotherapy (6), however, PB contamination can lead to misdiagnosing CNS involvement as the origin of a malignant population detected in a contaminated CSF sample remains unclear. Another pitfall can be the rapid decay of the cells in a CSF sample especially if the CSF sample has to be transferred to a central laboratory e.g. for flow cytometry. As the degradation rate is different in each cell type, long transfers of the sample will affect the qualitative analysis and pathological cell populations might be missed. Low viable cell count is usually not a problem with PB and BM samples but CSF samples are frequently paucicellular, further hampering the analysis. Another hurdle is that there is no consensus on either the minimal number of cells needed for adequate analysis (varying between 100 and 1000 in literature) (7,8), or on the precise definition of a traumatic tap (PB contamination) (9,10,11).

Overall, obtaining correct results in diagnostic and follow-up samples of a childhood ALL or any other leukemia/lymphoma depends on the quality of the BM/CSF sample to a great extent.

We aimed to explore the percentage of inadequate BM/CSF samples when we could not interpret and report the results owing to preanalytical errors.

## PATIENTS AND METHODS

**Cohort 1a:** data of patients with childhood ALL between 2011 and 2018 were analyzed retrospectively. Day 15 bone marrow samples were obtained from 104 patients, 59% of whom were male, 41% were female. 23 patients (20 male, 3 female) had T-ALL (22%), 81 patients (41 male, 40 female) had BCP-ALL. Average age at sampling

**Table 1** Antibody panels and clones used for staining the childhood day 15 and day 33 BM samples

A	FITC	PE	PerCP-Cy5.5	APC
Tube 1	CD20 (L27) <sup>1</sup>	CD10 (SS2/36) <sup>3</sup>	CD34 (8G12) <sup>1</sup>	CD19 (SJ25C1) <sup>1</sup>
Tube 2	CD20+CD10 (L27+SS2/36) <sup>1,3</sup>	CD38 (HB7) <sup>1</sup>	CD45 (2D1) <sup>1</sup>	CD19 (SJ25C1) <sup>1</sup>
Tube 3	CD58 (AICD58) <sup>2</sup>	CD10 (SS2/36) <sup>3</sup>	CD45 (2D1) <sup>1</sup>	CD19 (SJ25C1) <sup>1</sup>
Tube 4	cyFXIII-A	CD10 (SS2/36) <sup>3</sup>	CD45 (2D1) <sup>1</sup>	CD19 (SJ25C1) <sup>1</sup>
Tube 5	syto16	CD10 (SS2/36) <sup>3</sup>	CD45 (2D1) <sup>1</sup>	CD19 (SJ25C1) <sup>1</sup>

B	FITC	PE	PerCP-Cy5.5/PE-Cy5.5	PE-Cy7	APC	APC-H7	PB	PO
Tube 1	CD58 (AICD58) <sup>2</sup>	CD123 (SSDCLY107D2) <sup>2</sup>	CD33 (D3HL60.251) <sup>2</sup>	CD19 (J3-119) <sup>2</sup>	CD10 (HI10a) <sup>1</sup>	CD81 (JS-81) <sup>1</sup>	syto40 <sup>7</sup>	CD45 (HI30) <sup>5</sup>
Tube 2	syto16 <sup>7</sup>	CD66c (KOR-SA3544) <sup>2</sup>	CD34 (8G12) <sup>1</sup>	CD19 (J3-119) <sup>2</sup>	CD10 (HI10a) <sup>1</sup>	CD38 (HB7) <sup>1</sup>	CD20 (2H7) <sup>4</sup>	CD45 (HI30) <sup>5</sup>
Tube 3	cyFXIII-A	CD10 (SS2/36) <sup>3</sup>	CD45 (2D1) <sup>1</sup>	-	CD19 (SJ25C1) <sup>1</sup>	-	-	-

C	FITC	PE	PerCP-Cy5.5	APC
Tube 1	syto16	CD7 (8H8.1) <sup>2</sup>	CD45 (2D1) <sup>1</sup>	CD3 (SK7) <sup>1</sup>
Tube 2	CD4 (SK3) <sup>1</sup>	CD8 (SK1) <sup>1</sup>	CD45 (2D1) <sup>1</sup>	CD3 (SK7) <sup>1</sup>
Tube 3	CD99 (DN16) <sup>6</sup>	CD7 (8H8.1) <sup>2</sup>	CD5 (L17F12) <sup>1</sup>	CD3 (SK7) <sup>1</sup>
Tube 4	nTdT (HT6) <sup>3</sup>	CD7 (8H8.1) <sup>2</sup>	CD3 (SK7) <sup>1</sup>	cyCD3 (SK7) <sup>1</sup>

D	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7	PB	PO
Tube 1	syto16 <sup>7</sup>	CD99 (3B2-TA8) <sup>5</sup>	CD3 (SK7) <sup>1</sup>	CD7 (8H8.1) <sup>2</sup>	CD1a (HI149) <sup>1</sup>	CD8 (SK1) <sup>1</sup>	CD4 (RPA-T4) <sup>1</sup>	CD45 (HI30) <sup>5</sup>
Tube 2	nTdT (HT6) <sup>3</sup>	CD99 (3B2-TA8) <sup>5</sup>	CD5 (L17F12) <sup>1</sup>	CD7 (8H8.1) <sup>2</sup>	cyCD3 (SK7) <sup>1</sup>	CD3 (SK7) <sup>1</sup>	syto40 <sup>7</sup>	CD45 (HI30) <sup>5</sup>

**A:** 4-colour BCP-ALL panel for MRD detection; **B:** 8-colour BCP-ALL panel for MRD detection. From September, 2017, CD123 was added to Tube 2 (as CD66c+CD123 in FL2) and replaced by CD73+CD304 (clones: ad2 and 12C2, respectively, both manufactured by SONY Biotechnology, San José, CA, USA) in Tube 1; **C:** 4-colour T-ALL panel; **D:** 8-colour T-ALL panel. See Abbreviations for the fluorochromes. Manufacturers: 1: Becton-Dickinson Pharmingen, San José, CA, USA; 2: Beckman-Coulter, Brea, CA, USA; 3: Dako, Glostrup DK; 4: BioLegend, San Diego, CA, USA; 5: ExBio, Praha, CZ; 6: AbDSerotec, Raleigh, NC, USA; 7: Molecular Probes, Eugene, OR, USA. cyFXIII-A is a homemade antibody (13)

time of the whole population was 83 months, with a range between 1 and 201 months.

**Cohort 1b:** day 33 bone marrow samples were analyzed from 90 patients (56% male, 44% female), 13 (14%) T-ALL (11 male, 2 female), 77 (86%) BCP-ALL (39 male, 38 female). Average age in this population was also 83 months, range between 2 and 202 months.

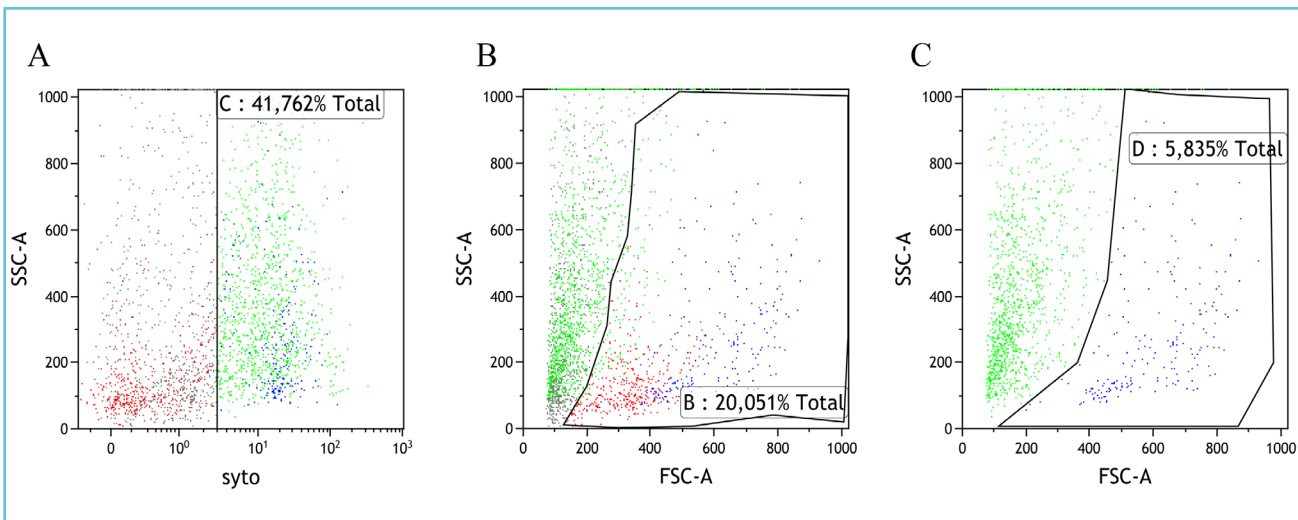
**Cohort 2:** in the mentioned time period a total of 26 CSF samples were analyzed by flow cytometry from 20 pediatric patients with ALL. The average age was 75 months, range between 7 and 214 months. Twelve patients were male (60%), eight were female (40%). One patient had T-ALL (5%), the others had BCP-ALL (95%). More than one sample was sent from five patients, 3 samples from the T-ALL patient and two samples each from the other four patients.

**Cohort 3:** fifty-one CSF samples from 47 patients (adults and children) were evaluated and viable cell percentage in native and stabilized samples (TransFix<sup>®</sup>; Ref. No. TF-CSF-5-25, Caltag Medsystems, Buckingham, UK) were compared. Nineteen of these 47 patients were female (40%), 28 were male (60%). Average age in this group was approximately 43 years with a range of 10 months and 79 years. 29 samples were stabilized, 22 were native.

Flow cytometric measurements were carried out in an 8-colour FACSCanto II flow cytometer, data were analyzed by FACSDiva 8.0.2 software (both by Beckton Dickinson Biosciences, San Jose, CA, USA). Pediatric ALL samples before March, 2013 (regarding BCP-ALL) and September, 2013 (regarding T-ALL) were examined in a 4-colour setting, all samples afterwards were examined by 8-colour setting, labeled in a stain-lyse fashion. The labeling procedure was performed as previously described (12) Antibody panels with clones and manufacturers are summarized in Table 1. **Cohort 2** CSF samples were stained with antibodies based on these panels; due to sample shortage in most cases the whole panels could not be applied. To make the results comparable, the flow cytometer was calibrated daily, using Cytometer Setup and Tracking fluorescent microbeads (Cat No. 641319, Becton Dickinson Biosciences, San Jose, CA, USA) and Autocomp software as recommended by the manufacturer.

Viable cell count in bone marrow samples was evaluated by syto-staining. Duplicates were excluded from all samples before the evaluation of the percentage of syto+ viable cells. Bone marrow samples containing < 2% erythroid precursors were considered contaminated by PB

**Figure 1** Syto+FSC/SSC sequential gating of CSF samples to evaluate viable cells



Considerable difference was observed between Syto (A) and FSC/SSC gating (B) in viable cell percentage (example: Sample No. 3 from Cohort 2). Best result was achieved by Syto+FSC/SSC sequential gating (C).

according to the ALL IC-BFM 2009 Flow MRD SOP. In CSF samples viable cell count was evaluated either by syto+FSC/SSC sequential gating (Figure 1) or by FSC/SSC gating if syto was not used. CSF samples containing < 30% of viable cells were considered inadequate for analysis and PB contamination was concluded if the CSF sample contained > 100 red blood cells / microliter (RBC/ $\mu$ L).

## RESULTS

### Cohort 1a

Peripheral blood contamination was found in 16 (15%) out of 104 day15 BM samples (12 out of 81 BCP-ALL and 4 out of 23 T-ALL). Blast percentages in the BCP-ALL contaminated samples were all below the Flow High Risk limit (10%) therefore these samples were inadequate for flow risk stratification. In the T-ALL subgroup > 10% of residual blasts were found in 3 out of 4 contaminated samples, making them eligible for risk stratification as these patients belonged to the Flow High Risk group. Altogether 13 samples of day15 BMs could not be used for

risk stratification out of 104 samples (12.5%) owing to PB contamination.

### Cohort 1b

Thirteen samples (14%) out of 90 were contaminated with peripheral blood (11 out of 77 BCP-ALL and 2 out of 13 T-ALL). All these samples contained less than 10% blasts, so they were reported as inadequate.

### Cohort 2

RBC count was recorded in 22 out of the 26 samples. Details of this cohort are shown in Table 2. With 100 RBC/ $\mu$ L as cutoff for PB contamination 10 out of 22 (45%) samples were contaminated. Despite the low (< 10) white blood cell count / microliter (WBC/ $\mu$ L) in 11 samples (sample No. 1-7 and 9-12), several thousands of cells (1,840 – 100,000) could be acquired and analyzed by flow cytometry. Four out of the 11 paucicellular samples contained malignant cells (sample No. 9-12), one of them being PB contaminated (sample No. 12). Malignant cells were found in 14 samples from the overall 26, eight (57%) of which were PB-contaminated.

**Table 2** CSF data of *Cohort 2\**

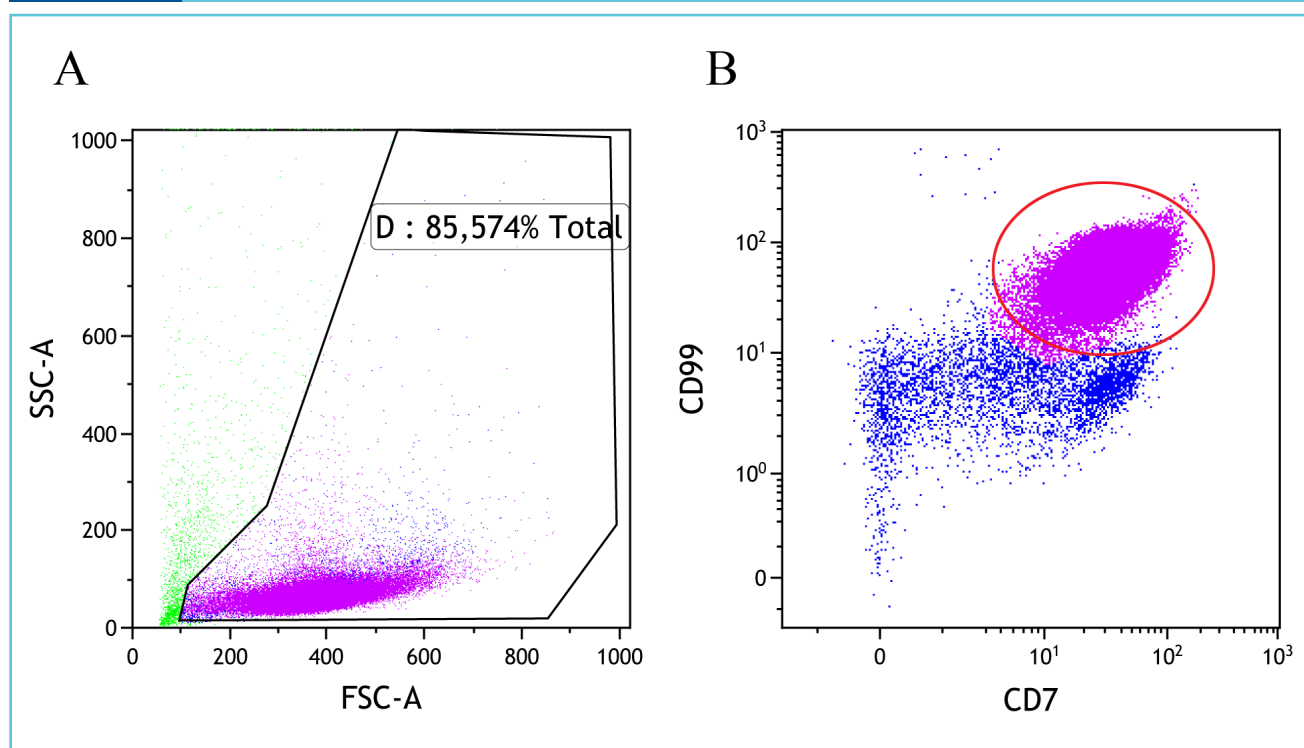
Sample no.	Viable cells <30%	PB contamination	Pathological cells	No. of events acquired	WBC/ $\mu$ L	RBC/ $\mu$ L
1	yes	-	-	10,000	2	11
2	yes	-	-	2,829	5	1
3	yes	-	-	3,496	6	1
4	yes	-	-	5,612	2	1
5	yes	-	-	2,024	4	N/A
6	yes	-	-	1,840	1	92
7	yes	yes	-	100,000	7	2,560
8	yes	yes	present	13,961	80	123
9	yes	-	present	2,576	4	N/A
10	yes	-	present	4,094	5	5
11	yes	-	present	9,246	8	8
12	yes	yes	present	3,887	9	1,280
13	yes	yes	present	76,245	336	24,533
14	yes	yes	present	2,576	40	8,533
15	-	yes	present	300,000	679	1,133
16	-	yes	present	300,000	12,800	107
17	-	yes	present	130,893	679	683
18	-	yes	present	3,082	140	90,453
19	-	yes	-	3,703	23	160
20	-	-	-	100,000	623	N/A
21	-	-	-	17,710	179	N/A
22	-	-	-	5,750	180	80



23	-	-	-	2,208	44	67
24	-	-	present	137,356	720	5
25	-	-	present	2,415	41	32
26	-	-	present	2,737	11	61

\* Samples 24-26 were from a patient with T-ALL, all other samples were from patients with BCP-ALL. N/A: not assessed.

**Figure 2** Example of a well-evaluable CSF sample of a patient with T-ALL\*



\* In Sample 24 (Cohort 2) viable cell percentage was 85.6% (A), among these cells 96% were pathological T lymphoblasts with bright CD99/CD7 expression (purple) (B).

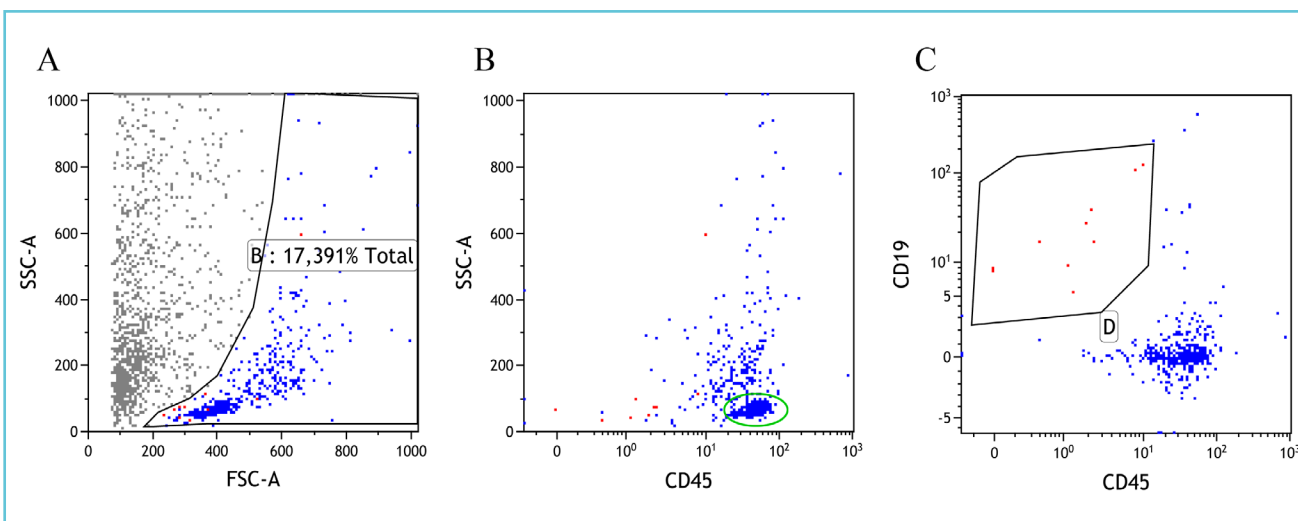
Figure 2 (above) shows dotplots of a non-PB contaminated, well-evaluable sample, with T-ALL blasts expressing bright CD99/CD7.

Eleven (42%) out of the 26 CSF samples were considered inadequate for evaluation due to < 30% of viable cells among the acquired nucleated cells, four of which had identifiable malignant cell population despite of the low percentage of viable cells (sample No. 8-11) (Figure 3).

### Cohort 3

Viable cell count was < 30% in 5 out of the 29 CSF samples (17%) in the subgroup with preservative and 9 out of 22 (41%) in the native subgroup (without stabilization), the difference is significant ( $p=0.05$ , Fisher's exact test). PB-contamination (> 100 RBC/ $\mu$ L) was found in 8 out of 22 samples (36%) in the native subgroup and in 4 out of 29 samples (14%) in the stabilized samples.

**Figure 3** Example of a poorly evaluable CSF sample of a patient with BCP-ALL\*



\*In Sample 9 (Cohort 2) viable cell percentage was below 30% (A) but a lymphocyte population was clearly visible (green circle) (B), along with malignant cells with CD45-/CD19+ characteristics (red dots, gate D) (C).

## DISCUSSION

Examining BM and CSF samples is essential in the diagnosis and follow-up of leukemias and lymphomas, including childhood ALL. Sample quality is a very important factor in obtaining adequate results. Hemodilution of a BM sample is quite common, His et al. found that 36% of the BM samples were hemodiluted from patients with acute leukemia (14).

According to the ALL IC BFM 2009 Flow MRD SOP a hemodiluted BM sample obtained on day 15 with the percentage of erythroid precursors below 2% is not eligible for flow risk stratification in childhood ALL (5). Such a hemodiluted sample is eligible for risk assessment only if the residual blast percentage is > 10%, meaning Flow High Risk. Those hemodiluted BM samples that contain < 10% residual blasts must not be used for risk stratification and the flow cytometry report must describe the sample as “inadequate”.

Several other methods of determining hemodilution in a bone marrow sample have been described (15,16,17). In our cohort (Cohort 1a) 12.5% of the day 15 BM samples of children

diagnosed with ALL were inadequate for risk assessment that might have hampered treatment adjustments in these cases. Similarly, 14% of the Day 33 BM samples (Cohort 1b) were also hemodiluted. Hemodilution can be best avoided if the BM aspiration is done prior to the biopsy and if no more than 1-2 mL of sample is obtained (18).

As hematological malignancies often affect the central nervous system, examination of the CSF is frequently needed. The core of the diagnosis is the identification of malignant cells by conventional cytomorphology in a CSF sample, although up to 60% of the cases can be false-negative (19,20). Flow cytometry has great sensitivity and specificity and is recommended by the National Comprehensive Cancer Network (USA) in conjunction with cytomorphologic studies (6). We could also confirm the utmost importance of flow cytometry, especially in cases of CSF samples with low nucleated cell count (< 10 WBC/ $\mu$ L) when malignant cells could be detected without PB contamination (samples No. 9-11 in Cohort 2). Kraan et al. suggested classifying cell clusters of > 25 cells as positive, 10-25 as suspicious and < 10 as negative (21). The cells decay



rapidly in the CSF after sampling with granulocytes and monocytes showing the highest rate of degradation (6). Several studies tried adding different types of cell culture media to improve cell survival with promising results (22,23).

Other methods were also examined, e.g. immediate cooling, minimizing centrifugation steps, aspirating supernatant instead of decanting sample (21,24,25). Preservatives (e.g. TransFix®) may also be used, moreover, TransFix® is recommended for CSF by the British Committee for Standards in Hematology (26). TransFix has been shown to stabilise malignant haematological cells in cerebrospinal fluid, making it possible to determine leucocyte subsets in CSF via flow cytometric analysis 72 hours after lumbar puncture (26,27). Previous studies showed that the use of TransFix/EDTA CSF Sample Storage Tubes prevents cellular loss and enhances flow cytometric detection of leptomeningeal localized hematological malignancies much better than serum-containing medium-filled tubes or untreated tubes, because scatter and antigen expression characteristics of pathological cells are preserved (28,29).

In our *Cohort 3*, significantly better results were achieved regarding the number of reportable results since more than 30% viable cells were detected in the CSF samples when TransFix® was used. However, pathological cells still can be identified in samples with low viable cell percentage, as it happened in the case of four samples (samples No. 8-11) in *Cohort 2*, therefore it is difficult to establish a clear cutoff for viable cell percentage under which the sample is considered inadequate and results are not reported. Reporting these cases should remain the decision of the examiner.

According to Petzold et al. up to 20% of standard lumbar punctures (LPs) are traumatic taps, although there is no consensus about the precise definition (30).

Some authors define a LP to be traumatic if at least 100 red blood cells (RBC) per microliter are present (31), others put the cutoff to 400 RBCs per microliter (32,33). However, according to Gajjar et al. a LP is defined traumatic if > 10 RBCs are present per microliter. They found that blood contamination in CSF in children with acute lymphoblastic leukemia had an adverse effect on treatment outcome (34).

According to Kraan et al. detecting a small malignant cell population in a contaminated CSF sample is diagnostic only if this malignant population is not detected in the peripheral blood obtained simultaneously (21). Te Loo et al. advise against performing a LP in phases of an acute leukemia when the frequency of malignant cells is high in the peripheral blood, as by a traumatic tap even the iatrogenic contamination of the CNS with the malignant cells can occur besides a false-positive CSF result (35).

In our two CSF cohorts, 45% and 24% of the samples were PB contaminated (*Cohort 2* and *Cohort 3* overall, respectively), which is in accordance with results found in the literature (30,36,37).

Limitations of the present results include the retrospective nature of the study, a prospective design would have enabled us to record all quality indicators of the preanalytical phase (38). Furthermore, our results concerning the percentage of reportable CSF samples with or without preservative need to be validated in higher number of samples as well.

In conclusion, poor sample quality can hamper risk stratification and further therapeutic decision in childhood ALL. Despite low viable cell count malignant cell populations may still be identified in a CSF sample, therefore establishing a certain cutoff point is difficult.



### **Abbreviations (in alphabetical order)**

**ALL:** acute lymphoblastic leukemia

**APC:** allophycocyanin

**APC-H7:** allophycocyanin – Hilite® 7

**B-CP-ALL:** B-cell precursor acute lymphoblastic leukemia

**CNS:** central nervous system

**CSF:** cerebrospinal fluid

**FITC:** fluorescein-isothiocyanate

**LP:** lumbar puncture

**PB:** peripheral blood

**PE:** Phycoerythrin

**PE-Cy5.5:** Phycoerythrin – cyanine 5.5

**PE-Cy7:** Phycoerythrin – cyanine 7

**PerCP-Cy5.5:** Peridinin-chlorophyll protein – cyanine 5.5

**PB:** Pacific Blue

**PO:** Pacific Orange

**RBC:** red blood cell

**T-ALL:** T-cell acute lymphoblastic leukemia



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