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B-Cell Precursors: Immunophenotypic Features in the Detection of Minimal Residual Disease in Acute Leukemia

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Abstract

Minimal residual disease (MRD) as a tool to monitor response to therapy is both a criterion for detailed risk stratification and an independent prognostic factor in childhood acute lymphoblastic leukemia (ALL). Immunological assays particularly flow cytometry (FC) are priority methods in MRD monitoring. Multicolor flow cytometry makes it possible to most fully characterize the immunophenotype of tumor B lymphoblasts and reveal leukemia-associated immunophenotypes not only according to the CD58 and CD38 antigens but also as an additional criterion of aberrancy. This allows you to identify and select individual criteria for further monitoring of minimal residual disease for each patient with ALL. The aim of this chapter is to compare immunophenotyping features of normal B-cell precursors and B-lymphoblasts in acute leukemia and to show possibilities of use of a leukemia-associated immunophenotype in monitoring of the MRD.

Keywords: bone marrow, B-cell precursors, acute leukemia, leukemia-associated immunophenotype, flow cytometry, minimal residual disease

1. Introduction

Control of response to chemotherapy is an intrinsic part of current treatment protocols [1–5]. Assessment of response in key points of chemotherapy programs helps both to stratify patients by risk groups more accurately and to avoid serious chronic side effects and patient overtreatment [6–12]. In case of acute leukemia, the number of tumor blasts undetectable in bone marrow (BM) morphologically at different therapy stages or minimal residual disease (MRD) is a criterion for such assessment.

In adult patients, problems of most significant points of immunological detection and the role of MRD levels are a matter of discussion [2]. The MRD significance is demonstrated in full in pediatric oncology. The MRD is of importance both in prognosis of acute lymphoblastic leukemia (ALL) and in prediction of recurrence [13]. Key points for MRD assessment are determined as well as their clinical significance and MRD levels that help in detailed risk stratification of patients [1].

Immunological quantification of MRD cells solves different problems depending on chemotherapy stages. In the middle of induction therapy (day 15), it evaluates primary response [14]. While at the end of induction therapy (day 33), the purpose is final risk stratification of patients with respect to clinical and immunological prognostic factors [1, 14, 15]. MRD assessment at the end of induction consolidation (day 78) identifies a patient group with so called slow response. These patients remain MRD-positive by days 15 and 33 and reach MRD-negativity by day 78 only. This group is characterized by good prognosis [3].

Many international research groups have developed flow cytometry (FC) protocols for MRD diagnosis; however, there is not a common approach yet. The St Jude Children’s Research Hospital (Memphis, USA) has proposed a simplified 3-color FC assay to detect MRD cells on day 15 of induction therapy [16] that is based on elimination of normal B-cell precursors (BCP) under the effect of corticosteroids [17] that are a basis for therapy at the given stage. The BFM international study group has developed an MRD monitoring protocol basing on the detection of B-LP with aberrant (leukemia-associated) immunophenotype (LAIP). Antigens CD58 and CD38 are most common markers to characterize the aberrant immunophenotype [18, 19]. However, tumor lymphoblasts show no aberrance by these antigens [20]. There are no clearly identified alternative combinations of aberrant markers.

In ALL from T-cell precursors (T-ALL), there are no clearly cut criteria for MRD assessment [21] and research in this field is ongoing.

The aim of this chapter is to compare immunophenotyping features of normal B-cell precursors and B-lymphoblasts in acute leukemia and to show possibilities of use of a leukemia-associated immunophenotype in monitoring of the MRD.

2. Materials and methods

The study involved 191 ALL cases (160 B-ALL [142—primary diagnosis, 18—diagnosis in relapse] and 31 T-ALL). The diagnosis was made basing on a combination of morphocytochemical and immunophenotyping assays of BM puncture biopsies.

In most cases (88.8% of B-ALL and 87.1% of T-ALL), the immunophenotyping at diagnosis was performed by 3-color FC (at least 20 markers was analyzed). The immunophenotyping using EuroFlow 8-color standardized panels was made in 11.2% of B-ALL and 12.9% of T-ALL cases. Lineage of blasts was determined using ALOT (acute leukemia orientation tube) (**Table 1**).

A more accurate B-ALL (**Table 2**) or T-ALL (**Table 3**) 8-color standardized panel was used depending upon the identified blast lineage. The BCP aberrance was assessed basing on expression of the following antigens: CD58, CD38, CD81, CD9, CD123, CD66c, CD13, CD33, CD20, CD21, and CD24; T-cell precursors (TCP) were characterized with respect to CD99, CD56 expression.

MRD quantification was made in 397 BM specimens from ALL patients at different therapy stages (days 15, 33, and 78, **Table 4**).

No./ fluorochrome	Markers							
	FITC	PE	PerCP-cy5.5	PE-cy7	APC	APC-117	V450	V500
1	cy MPO	cy CD79a	CD34	CD19	CD7	sm CD3	cy CD3	CD45

Table 1.
Acute leukemia orientation tube.

No./ fluorochrome	Markers							
	FITC	PE	PerCP-cy5.5	PE-cy7	APC	APC-117	V450	V500
1	CD58	CD66c	CD34	CD19	CD10	CD38	CD20	CD45
2	cyIgM	CD33	CD34	CD19	CD117 + sIgM	sIg-λ	sIg-κ	CD45
3	nuTdT	CD13	CD34	CD19	CD22	CD24	CD9	CD45
4	CD15 + CD65	NG2	CD34	CD19	CD123	CD81	CD21	CD45

Table 2.
EuroFlow consortium 8-color standardized panel for B-ALL diagnosis.

No./fluorochrome	Markers							
	FITC	PE	PerCP-cy5.5	PE-cy7	APC	APC-117	V450	V500
1	nuTdT	CD99	CD5	CD10	CD1a	smCD3	cyCD3	CD45
2	CD2	CD117	CD4	CD8	CD7	smCD3	cyCD3	CD45
3	TCRγδ	TCRαβ	CD33	CD56	cyTCRαβ	smCD3	cyCD3	CD45
4	CD44	CD13	HLA-DR	CD45RA	CD123	smCD3	cyCD3	CD45

Table 3.
EuroFlow consortium 8-color standardized panel for T-ALL diagnosis.

ALL immuno-subtype FC color number		MRD monitoring points								
		Day 15			Day 33			Day 78		
		3 col.	4–6 col.	8 col.	3 col.	4–6 col.	8 col.	3 col.	4–6 col.	8 col.
B-ALL	Total	139			148			54		
	No.	59	68	12	51	79	18	3	38	13
	Percentage	42.4	49.0	8.6	34.4	53.4	12.2	5.6	70.4	24.0
T-ALL	Total	30			26			—		
	No.	9	19	2	6	18	2	—	—	—
	Percentage	30.0	63.3	6.7	23.1	69.2	7.7	—	—	—

Table 4.
The number of analyses at each MRD monitoring point.

At diagnosis, every BM specimen was characterized both morphologically and immunologically. Myelogram count was made by two morphologists (250 cells each) on Giemsa stained BM smears. The following M-types were identified basing on standard morphological criteria for the number of blasts: M1—specimens with ≤5% of blasts, M2—specimens with 5.0–25.0% of blasts, and M3—specimens with ≥25% of blasts. After that, a more detailed analysis was made, and the morphological criteria were compared with immunological findings. Detailed analysis of MRD levels was made in M1, M2, and M3 specimens. Subgroups of specimens within each group were identified with respect to the number of MRD cells. The subgroups were identified according to the BFM protocol for MRD assessment on induction chemotherapy as follows: standard risk <0.1% (including negative cases), medium risk 0.1–10.0%, and high risk ≥10%/the level of 0.01% of tumor cells in a specimen was taken as a standard threshold for MRD negativity.

Statistical analysis was made using IBM-SPSS Statistics v.17 software. Parametric data were analyzed by comparison of means using Student's t-test. Comparison of nonparametric data was made by Pearson's χ^2 -test.

3. Results

3.1 General patient characteristics

A total of 186 patients with ALL (five patients were treated in debut and in relapse of the disease) managed at the N.N. Blokhin Cancer Research Center during 2006 through 2017 were entered in the study.

The B-ALL group included 155 patients 1–18 years of age: 78 boys (48.7%) and 82 girls (51.3%) (**Table 5**). In 142 cases (88.8%), the diagnosis was made at the disease onset. In 18 cases (11.2%), the diagnosis was made in recurrence stage. Five patients were followed up both in the debut and recurrences stages.

The T-ALL group consisted of 31 patients aged 2–17 years including 26 boys (83.9%) and 5 girls (16.1%) (**Table 6**).

Treatment was given according to the ALL IC BFM 2002 and 2009 protocols. Data on risk stratification are shown for 79 patients with B-ALL receiving treatment by the ALL IC BFM 2009 protocol (data for 63 patients with B-ALL onset are not available). The majority of patients (67.1%) were classified into an intermediate risk group. Only 5.1% of patients were a high risk group. And 27.8% of patients were assigned to a standard risk group.

71.0% of T-ALL patients received treatment by ALL IC BFM 2009 protocol. The majority of patients (72.7%) were classified into an intermediate risk group. Other patients (27.3%) were assigned to a high risk group.

Characteristic		Number	Percentage
Gender	M	78	48.4
	F	83	51.6
Age	1–18 years (mean 5.9 ± 0.3 years)		
Time of diagnosis	Debut	142	88.8
	Recurrence	18	11.2
Immunosubtype	Pre-pre-B	150	93.8
	Pro-B	10	6.2

Table 5.
Patient characteristics in the B-ALL group.

Characteristic		Number	Percentage
Gender	M	29	83.9
	F	7	16.1
Age	1–17 years (mean 7.3 ± 4.7 years)		
Time of diagnosis	Debut	31	100
	Pre-T-cell	12	38.7
	Cortico-thymocytic	19	61.3

Table 6.
Patient characteristics in the T-ALL group.

MRD monitoring was made on days 15, 33, and 78 of chemotherapy according to the treatment protocols used at the Research Institute of Pediatric Oncology and Hematology of the FBI N.N. Blokhin NMRCO and international recommendations.

3.2 ALL from B-cell precursors

3.2.1 Immunological characterization of blasts at primary diagnosis

Table 7 shows B-lymphoblast immunophenotype characteristics at primary diagnosis. These immunological characteristics were used further in MRD monitoring.

CD58 and CD38 are currently most commonly used antigens as main criteria of tumor B-lymphoblast aberrance. Blasts are considered aberrant if they are characterized by overexpression of CD58 in combination with low or no expression of CD38 (immunophenotype CD58⁺⁺CD38^{low/-}, **Figure 1**).

Analysis for this antigen combination demonstrated that blasts do not always have the CD58⁺⁺CD38^{low/-} immunophenotype, that is, aberrance by both antigens is found in 54.3% of cases only (**Table 8**). **Figure 2** shows an example of no aberrance by CD58 and CD38 antigens.

The following markers were studied as additional criteria of aberrance: CD123 (**Figure 3**), CD66c, CD81 (**Figure 4**), CD9, CD21, CD24 with their expression presented in **Table 9**. **Figures 3** and **4** show an example of aberrant expression of CD123 and CD81 in B-lymphoblasts at primary diagnosis.

3.2.2 Immunophenotypic characterization of blasts at relapse

A total of 18 patients were examined at relapse of the disease. Five patients were examined at both disease onset and relapse. Blast characterization was made using a

Antigen	No. of cases analyzed	Frequency of Ag-positive cases (%)
CD10	142	93.0
CD34	139	74.1
nuTdT	24	100.0
smCD22	137	50.3
CD20	129	14.7
Pan-myeloid (CD13, CD33)	137	46.0
CD58 ⁺⁺ CD38 ^{low/-}	50	54.0
CD38 ^{+/++}	93	54.8
Additional criteria of aberrance		
CD123	8	100.0
CD66c	8	75.0
CD81	7	14.3
CD9	8	87.5
CD21	7	0.0
CD24	7	100.0

Table 7. Tumor blast immunophenotype characteristics at primary diagnosis at disease onset.

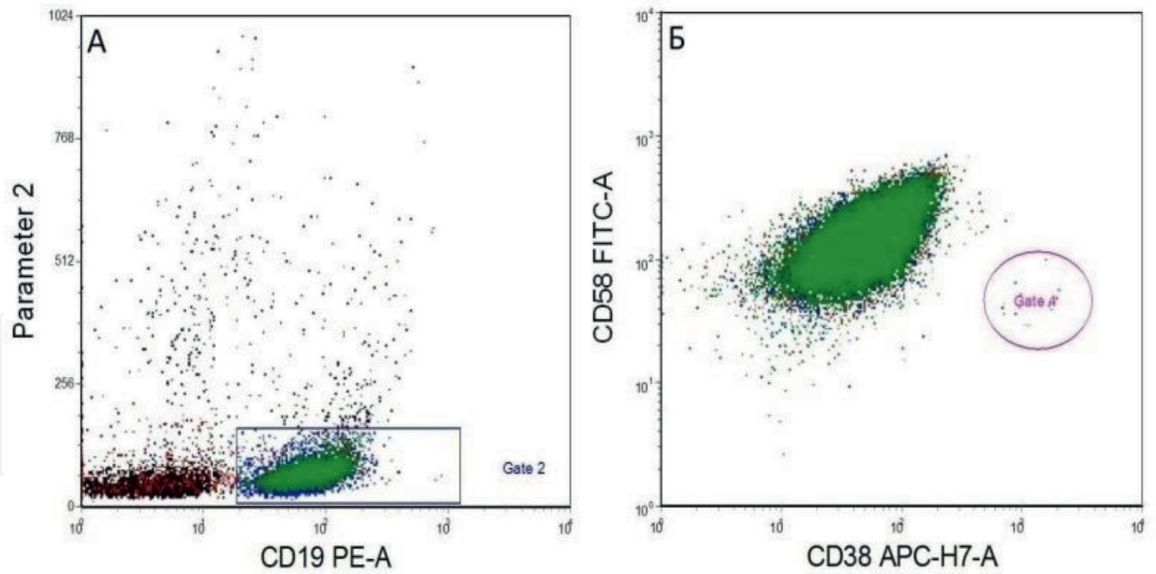


Figure 1.

Blasts with aberrant immunophenotype $CD58^{++}/CD38^{low}$. Cytogram A: blasts (gate 2, green) demonstrate bright CD19 expression (X-axis) against side scattering parameters (SSC, Y-axis). Cytogram B: B-lymphoblasts demonstrate bright CD58 expression (Y-axis) in combination with low CD38 expression (X-axis) as compared to normal BCP ($CD58^{+}CD38^{+}$, gate 4).

CD58/CD38 expression	Number	Percentage
$CD58^{++}/CD38^{low/-}$	25	54.3
$CD58^{+}/CD38^{low/-}$	2	4.3
$CD58^{++}/CD38^{+}$	8	17.4
$CD58^{+}/CD38^{+}$	11	24.0
Total	46	100.0

Table 8.

Blast immunophenotypes with respect to CD58 and CD38 expression at primary diagnosis.

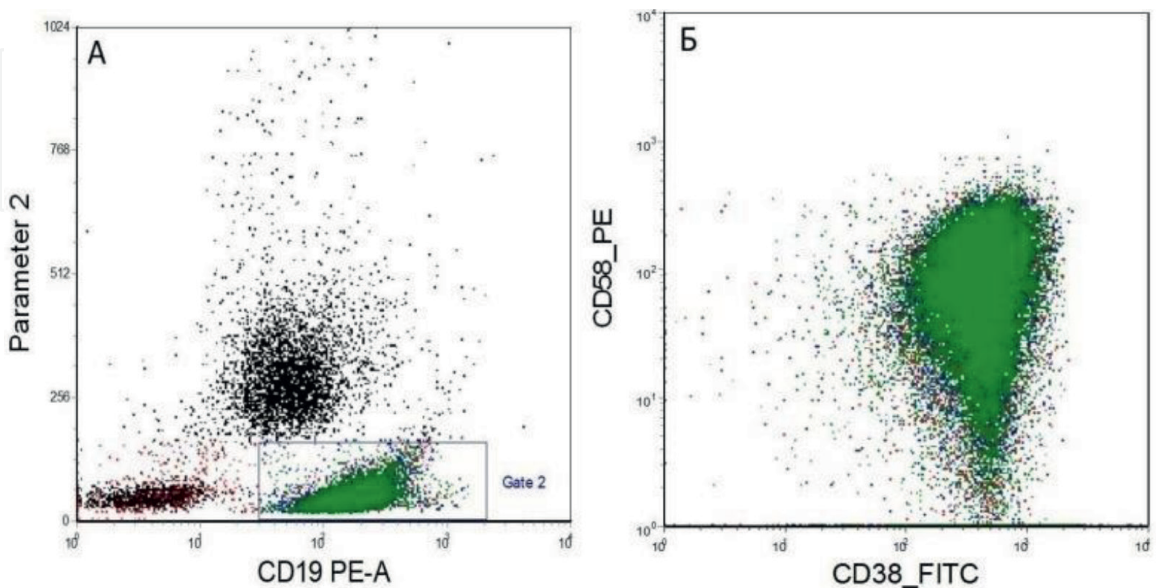


Figure 2.

Tumor B-lymphoblasts with immunophenotype $CD58^{+}/CD38^{+}$. Cytogram A: tumor B-lymphoblasts are identified basing on CD19 expression (X-axis) and low SSC characteristics (Y-axis). Blasts ($CD45^{low}CD19^{+}$) are characterized by bright CD38 expression (X-axis) in combination with normal CD58 expression (Y-axis) (cytogram B).

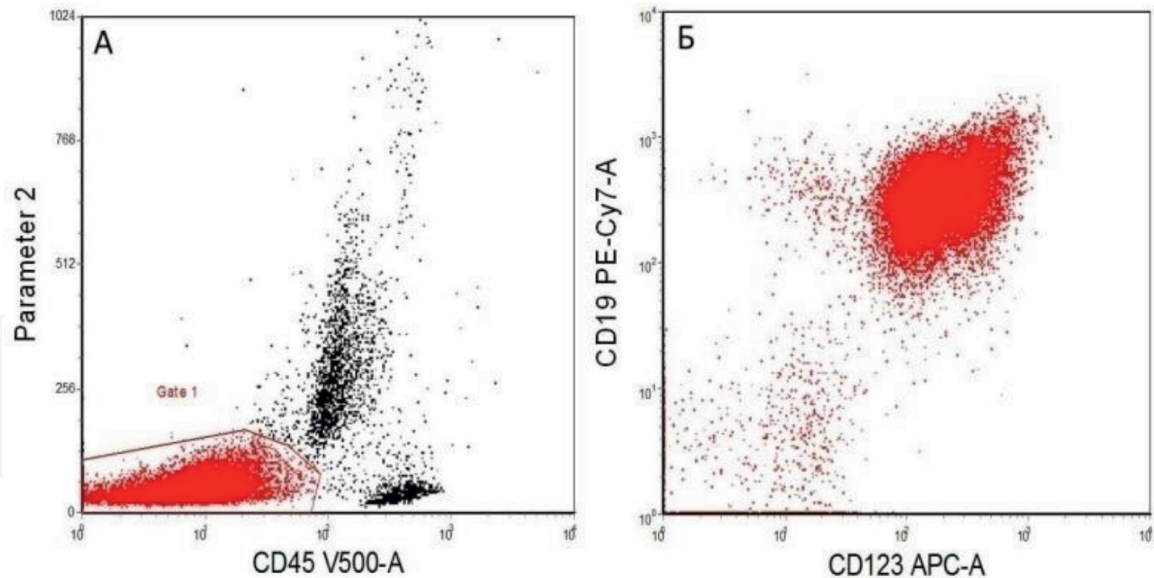


Figure 3.
Tumor B-lymphoblast aberrance by CD123 at primary diagnosis. Cytogram A: blasts (gate 1, red) are detected by low CD45 expression (X-axis) and low SSC characteristics (Y-axis). Cytogram B: tumor lymphoblasts demonstrate bright expression of pan-B-cell antigen CD19 (Y-axis) in combination with bright (aberrant) CD123 expression (X-axis).

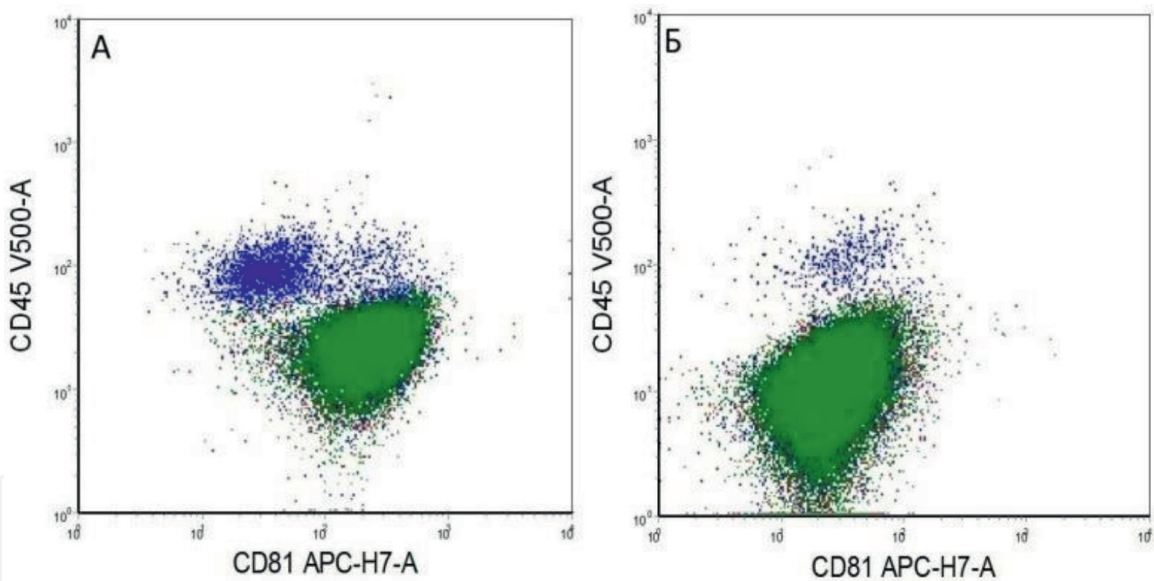


Figure 4.
Normal (A) and aberrant (B) expression of CD81 on B-LP. Cytogram A: normal bright CD81 expression on tumor B-lymphoblasts (green) in X-axis (CD81) against Y-axis (CD45) coordinates. Cytogram B: tumor B-lymphoblasts (green) with aberrant (weak) CD81 expression in X-axis (CD81) against Y-axis (CD45).

3-color FC protocol in 66.7% (12/18) patients. In six patients (33.3%), immunophenotyping used the EuroFlow consortium 8-color protocol.

Expression of main CD58/CD38 aberrance markers at disease recurrence was assessed in 13 patients.

Blast immunophenotype with respect to CD58 and CD38 expression intensity as compared with the primary diagnosis changed in 2 of 5 cases. In the first case, blasts at primary diagnosis were immunophenotypically close to normal BCP as concerns CD58 and CD38 expression ($CD58^+CD38^{++}$), while acquiring aberrant CD58 and CD38 expression ($CD58^{++}CD38^+$) at relapse. In the second case, on the opposite, blasts lost aberrant CD38 sign and became close to normal non-tumor B-lymphoblasts by this antigen expression.

Antigen	No. of cases studied	Frequency of Ag-positive cases (%)
nuTdT	31	80.6
CD34	31	19.4
CD10	31	38.7
CD4 ⁺ CD8 ⁺	31	35.5
CD1a	31	54.8
CD5	31	90.3
CD7	31	96.8

Table 9. Detection frequency of antigens associated with various TCP differentiation stages in T-ALL.

3.2.3 MRD assessment on day 15 of therapy

We compared morphological and immunological findings from BM analysis on day 15 of therapy. In group M1, morphological and FC data were fully similar (no blasts by morphology and complete MRD negativity [MRD cells < 0.01%] by FC) in 11.5% of cases.

Specimens from the M1 group were characterized by a marked percentage of lymphocytes (mean > 60.0%). The presence of MRD-positive specimens (FC) in the M1 group (good morphological response) may be explained by blast mimicry, that is, similarity with lymphocytes. This makes difficult accurate morphological verification of residual leukemic blasts.

In group M1, there was no contradiction between morphological and FC data. Most M3 specimens (85.7%) contained $\geq 10.0\%$ of MRD-positive cells by FC. The fraction of MRD-positive cells was 5.5% in one case only; that is, in terms of FC, this patient might be referred to the intermediate risk group and did not require therapy intensification.

Comparison of two FC protocols for MRD monitoring on day 15 (3-color St. Jude [28] based on identification of CD10⁺/CD34⁺ BCP among BM mononuclear fraction, and BFM protocol based on identification of BCP with aberrant immunophenotype among nucleated cells [NC]) demonstrated that the mononuclear approach was a more strict criterion for risk stratification of patients.

MRD on day 15 of induction therapy in B-ALL is always represented by BCP. Their identification is based on detection of CD10 and CD34 expression or no expression of pan-leukocyte antigen CD45 and combination of the above mentioned markers in B-cells. Quantification of MRD cells on the basis of each of the progenitor markers demonstrated that CD10 was the most reliable criterion for evaluation of BCP cells on day 15 of chemotherapy in children with pre-pre-B ALL. While in pro-B B-ALL, the count of BCLP should be made basing on nuTdT-positive cyCD22-expressing B-cells.

CD58/CD38 expression was assessed on day 15 of induction chemotherapy in 28 patients. All BCP, that is, MRD cells demonstrated aberrant immunophenotype CD58⁺⁺/CD38^{low} only in 15 of 28 patients (54%) (**Figure 5**).

CD58⁺⁺/CD38^{low/-} BCP content was analyzed in patients with no aberrance by this antigen combination at primary diagnosis (n = 11). The proportion of CD10⁺ B-cells was similar to that of CD58⁺⁺/CD38^{low/-} BCP in four specimens only. This example is illustrated in **Figure 5**. In the remaining cases, the number of CD58⁺⁺/CD38^{low} BCP was less than the number of CD10⁺ B-cells.

The analysis demonstrated a significant predominance of MRD on day 15 of therapy in pro-B ALL as compared to pre-B (p = 0.016).

Day 15 of induction chemotherapy is characterized by BM hypocellularity and a marked fraction of debris that can interfere with results (**Figure 6**). To avoid this,

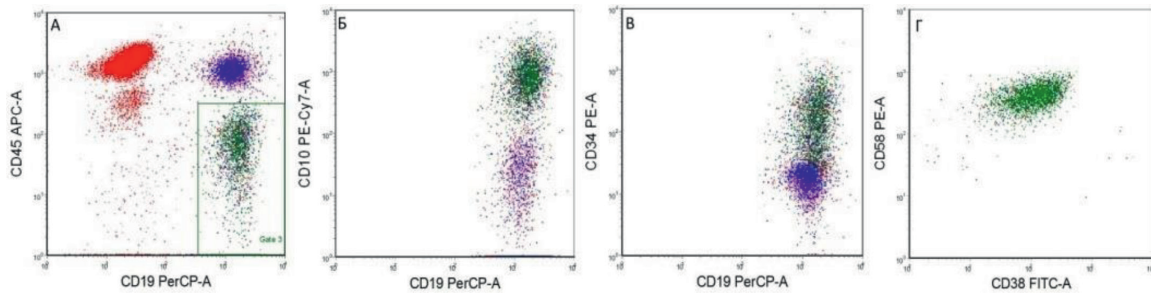


Figure 5. BCP immunophenotype features on day 15 of induction therapy. Cytogram A: 16.6% CD19⁺CD45^{low} BCP (gate 3, green) in CD19 (X-axis) against CD45 (Y-axis) coordinates. The cells demonstrate bright CD10 (cytogram B), CD19 (X-axis) expression against CD10 (Y-axis) and marked CD34 expression (cytogram C), CD19 (X-axis) against CD34 (Y-axis). MRD cells show aberrant immunophenotype CD58⁺⁺CD38^{low} (cytogram D, CD38 [X-axis] against CD58 [Y-axis]).

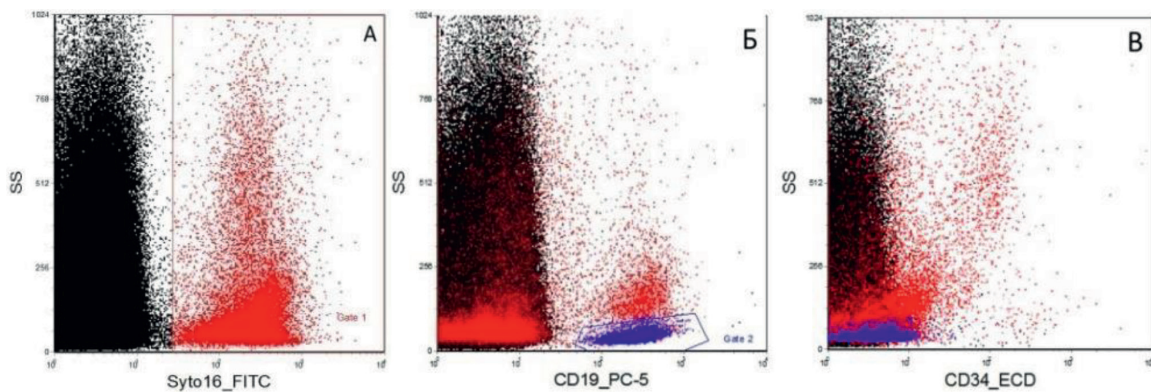


Figure 6. Day 15 of induction therapy. The specimen has much debris. Cytogram A: NC detection basing on expression of nucleotropic die Syto16 against SSC. The specimen has 3.65% of NC (gate 1, red). Cytogram B: evaluation of B-cell fraction (gate 2, blue): CD19 (X-axis) against SSC. The B-cell fraction without NC is 0.57%, with recount for NC CD19⁺ cells are 15.6% in this specimen. CD34⁺ BCP are 0.0021% without NC, that is, MRD-negativity. Recount with respect to Syto16 results in CD34⁺ BCP being 0.06%, that is, MRD-positivity.

a nucleotropic Syto die (Sito16 and Sito16 in our study) was included in the MRD detection panel.

In the group, as a whole MRD-negative status was found on day 15 of induction chemotherapy in 11.5% of patients, which allowed reduction in anthracycline dose to be considered with respect to clinical prognostic factors without the risk of recurrence in this subgroup.

3.2.4 MRD assessment on day 33 of therapy

MRD quantification was based on count of CD58⁺⁺CD38^{low/-} BCP. If specimens demonstrated no aberrance by this antigen combination at primary diagnosis, MRD was assessed by CD58⁺⁺CD10⁺⁺ or CD38^{low}CD10⁺⁺ combinations.

According to the morphological risk stratification criteria, all specimens contained less than 5.0% of blasts (1.2% ± 0.1, n = 80) and were included in the M1 group. There were no blasts in 6.25% of specimens (5/80). MRD-positivity by immunology was identified in 40.0% of cases (32/80). In two patients (2.5%), MRD cells were >1.0%. There were no high risk specimens (MRD ≥ 10.0%) in our study.

CD58 and CD38 primary tumor aberrance on day 33 of induction chemotherapy was assessed in 50.0% of patients. CD58 aberrance was found in 28 of 40 cases (70%), and CD38 aberrance was detected in 60.0% of cases (24/40). 55.0% of cases (22/40) were CD58/CD38 aberrant and 45.0% (18/40) demonstrated no aberrance.

Cases without CD58/CD38 aberrance at the primary diagnosis were examined for markers identifying cells of residual tumor by the literature.

We analyzed CD81, CD123, CD9, CD21, CD24 expression in 21 BM specimens. As a result, most informative marker combinations for detection of aberrant B-lymphoblasts were identified: combinations of CD58 or CD38 with CD10 (aberrant immunophenotypes $CD58^{++}CD10^{+}$ or $CD38^{+}CD10^{++}$) were the most informative in terms of FC. In case of CD20-positive ALL MRD could be detected by asynchronous CD20 and CD34 expression (aberrance characterized by $CD34^{+}CD20^{+}$ phenotype, **Figure 7**). In most cases, residual tumor may be detected by marked expression of CD123 in combination with weak or no expression of CD81 (aberrant immunophenotype $CD123^{++}CD81^{low}$, **Figure 8**). In some cases, CD9 monomorphic tumor expression especially in combination with one of clear-cut

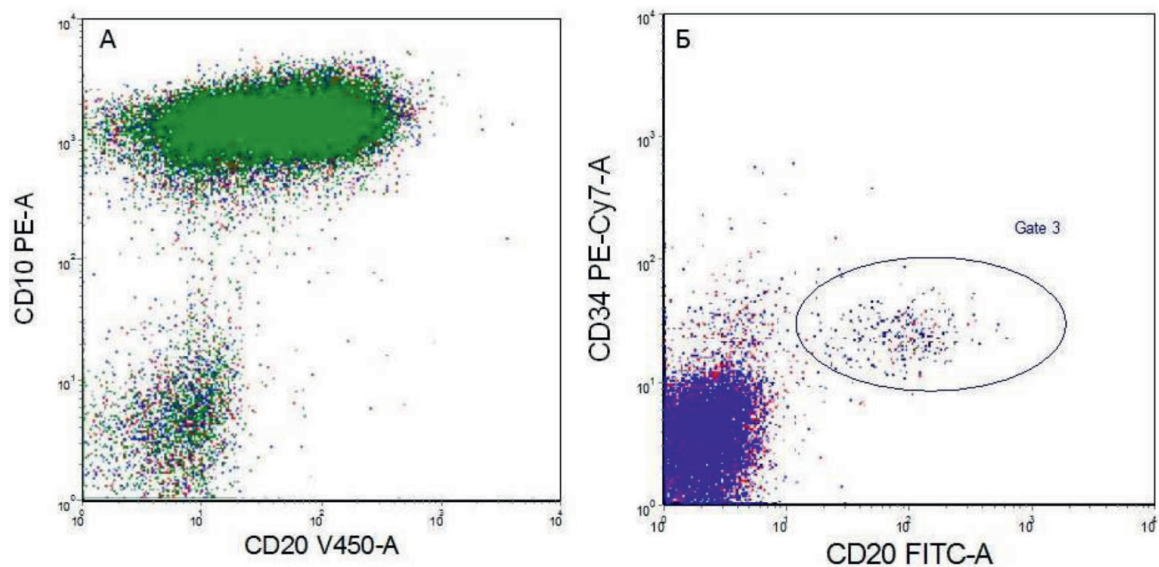


Figure 7.

MRD quantification basing on asynchronous expression of stage-specific Ag CD34/CD20 in a patient with $CD20^{+}$ pre-pre-B B-ALL immunosubtype. Cytogram A: tumor B-lymphoblasts ($CD45^{low}CD19^{+}$) at primary diagnosis (X-axis CD20 against Y-axis CD10), of which 60.0% express CD20. Cytogram B: MRD detection basing on simultaneous CD20 (X-axis) and CD34 (Y-axis) expression. MRD cells are 0.027% of NC specimen, that is, MRD-positivity.

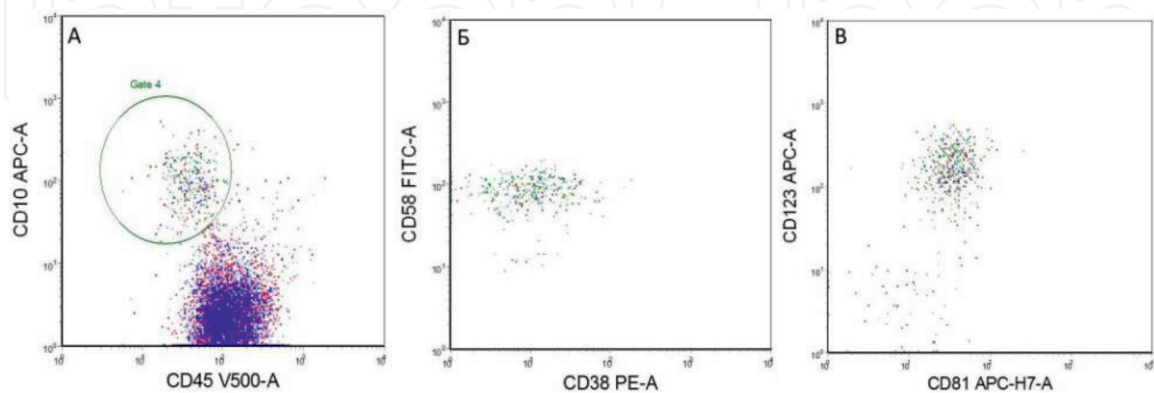


Figure 8.

MRD quantification basing on aberrance by CD58/CD38 and CD123/CD81. Cytogram A: BCP detection basing on bright CD10 (Y-axis) expression in combination with low CD45 expression (X-axis), gate 4 (0.03% of NC) within $CD19^{+}$ B-cells. Cytogram B: BCP are characterized CD58 overexpression (Y-axis) in combination with no CD38 expression (X-axis) and demonstrate $CD123^{++}CD81^{low}$ immunophenotype (cytogram C: CD123 [Y-axis] against CD81 [X-axis]). Therefore, B-lymphoblasts are aberrant by all four Ag analyzed, and there are no normal BCP in this specimen. Aberrant BCP are 0.027% of NC specimen, that is, MRD-positivity.

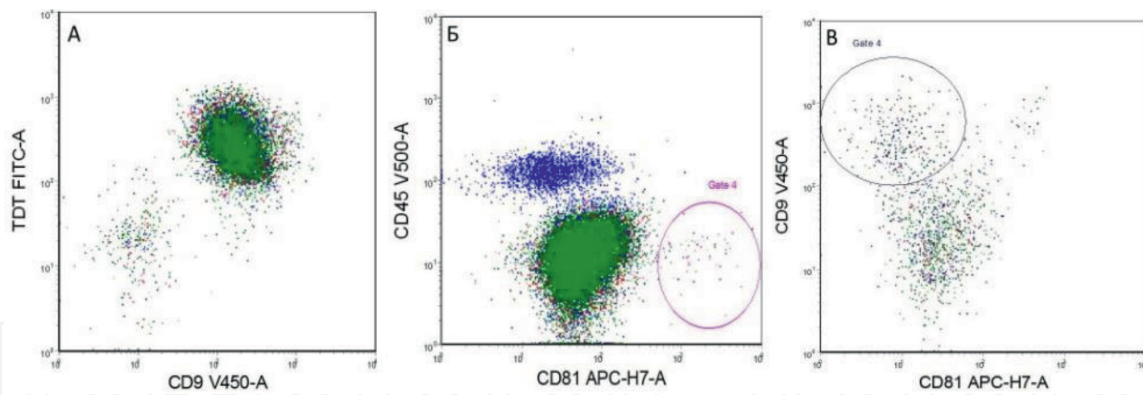


Figure 9. MRD quantification basing on aberrance by CD9 and CD81. Cytogram A: tumor B-lymphoblasts ($CD45^{low}CD19^{+}$) at primary diagnosis with bright nuTdT expression (Y-axis) in combination with monomorphic CD9 overexpression (X-axis). Cytogram B: tumor cells are aberrant by CD81 (X-axis CD81 against Y-axis CD45). As compared to residual normal BCP (gate 4, pink) with bright CD81 expression, tumor B-lymphoblasts demonstrate low (aberrant) Ag expression. Cytogram C: MRD detection basing on bright monomorphic CD9 overexpression (Y-axis) in combination with low CD81 expression (X-axis) (gate 4, dark blue). They are 0.015% of NC specimen: MRD-positivity.

aberrance criteria ($CD58^{++}$, $CD123^{++}$, $CD38^{low}$, $CD81^{low}$, **Figure 9**) are a sign of tumor aberrance and criterion for MRD assessment.

In cases with monomorphic co-expression of myeloid antigens (CD66c, CD13 or CD33), MRD assessment may be based on these markers.

Of the whole patients, cohort (80 patients) MRD-negative status was determined by FC in 60.0% (48 patients), and 40.0% (32 patients) were found MRD-positive. Comparison of basic morphological and immunological characteristics in these groups identified significant differences in the number of blasts ($p = 0.012$) and $CD19^{+}$ B-cells ($p = 0.044$).

Basing on FC quantification of MRD, most patients (65.0%) receiving treatment by ALL IC BFM 2009 protocol were included in the intermediate risk group.

3.2.5 MRD assessment on day 78 of therapy

MRD assessment on day 78 of therapy was made in 42 patients and was based on detection of BCP with aberrant immunophenotype ($CD58^{++}CD38^{low/-}$).

As recommended by BFM protocols, BM specimens were divided into MRD-positive and MRD-negative subgroups. Most BM specimens were MRD-negative ($n = 27$), and 15 of 42 specimens (35.7%) were MRD-positive.

In 14 patients, MRD status was assessed at three points of treatment protocol (days 15, 33 and 78). Seven of them achieved MRD-negativity by day 78 and met the criterion of slow response to therapy.

3.2.6 MRD assessment after 1 block of anti-relapse therapy (day 35)

After 1 block of anti-recurrence therapy, MRD was detectable in 15 BM specimens. By FC, 40.0% of patients ($0.004 \pm 0.0008\%$, $n = 6$) were MRD-negative and 60.0% of patients ($7.3 \pm 4.8\%$, $n = 9$) were MRD-positive with 77.0% of the MRD-positive specimens ($0.9 \pm 0.6\%$, $n = 7$) containing $<5.0\%$ of blasts by morphological study.

3.2.7 MRD monitoring on targeted therapy

In our study, one patient with early recurrence of ALL (pre-pre-B immunosubtype) received anti-recurrence targeted agent blinatumomab (biospecific

anti-CD19 monoclonal antibody). Blinatumomab mechanism of action involves specific binding with CD19⁺ cells and recruitment of effector T-cells to enhance response. According to the literature data, CD19 is not expressed on cells during blinatumomab therapy which makes difficult BCP identification. We chose an alternative BCP identification procedure to monitor MRD in this patient category: BCP identification and characterization on blinatumomab (anti-CD19) therapy should be based on expression of cyCD22 and nuTdT due to lost of CD19 expression.

3.3 ALL from T-lineage precursors

A total of 31 patients with T-ALL were included in our study. About 61.3% of patients (n = 19) presented with cortico-thymocytic immunosubtype (CD1a expression and/or the presence of CD4⁺CD8⁺ population) as determined by primary immunophenotyping. **Table 9** shows characteristics of blast immunophenotype at the stage of primary diagnosis.

Since T-cell ontogenesis is characterized by the absence of T-cell progenitors in normal BM, MRD monitoring by FC at all therapy stages involves detection and count of TCP.

According to BFM protocol, MRD detection in T-ALL is based on identification of T-cells with aberrant immunophenotype within CD7-positive fraction. However, CD7 demonstrates a broad range of expression and is present not only on TCP (NK-cells and myeloid progenitor cells); therefore, a cytometric protocol for TCP detection in BM (MRD cells) was developed that was based on expression of pan-T-cell antigen CD3 in cytoplasm (cyCD3) as the most stable lineage-specific pan-T-cell antigen. Choice of appropriate antigen clone is an important requirement for detection of cytoplasmic CD3 expression. For instance, SK7 clone is used to detect membrane determinant, while UCHT-1 binds to cytoplasmatic determinant. So, only UCHT-1 monoclonal antibody should be used in analysis of cyCD3.

In our study, quantification of TCP in BM was based on detection of subpopulations smCD3⁻CD7⁺, smCD3⁻TdT⁺, smCD3⁻CD1a⁺, and CD4⁺CD8⁺ within cyCD3⁻ positive BM T-cells at all time points of MRD monitoring.

To simplify recount and exclusion of debris, the quantification was made within NC of specimens as identified by Syto dies. The highest values were used as recommended by MRD detection protocols.

3.3.1 MRD assessment on day 15 of therapy

Quantification of residual blasts on day 15 of induction chemotherapy was made in 30 BM specimens.

The morphological and immunological findings were as a whole similar. The group M2 was heterogeneous demonstrating variability in blast numbers and MRD levels; however, these specimens belonged to intermediate risk group both by M-subtypes and MRD levels and therefore did not interfere in patient stratification.

BM characteristics on day 15 of treatment were analyzed with respect to T-ALL immunosubtype. The pre-T immunosubtype was characterized by significantly higher MRD level on day 15 as compared to cortico-thymocytic one (p = 0.044).

About 76.2% of patients composed the intermediate risk group (according to ALL-IC-BFM 2009 protocol), and 23.8% were stratified into the high risk group. There were no patients meeting criteria for standard risk in our study

Basing on MRD level on day 15 of induction chemotherapy, 25.0% were transferred from the intermediate to the high risk group.

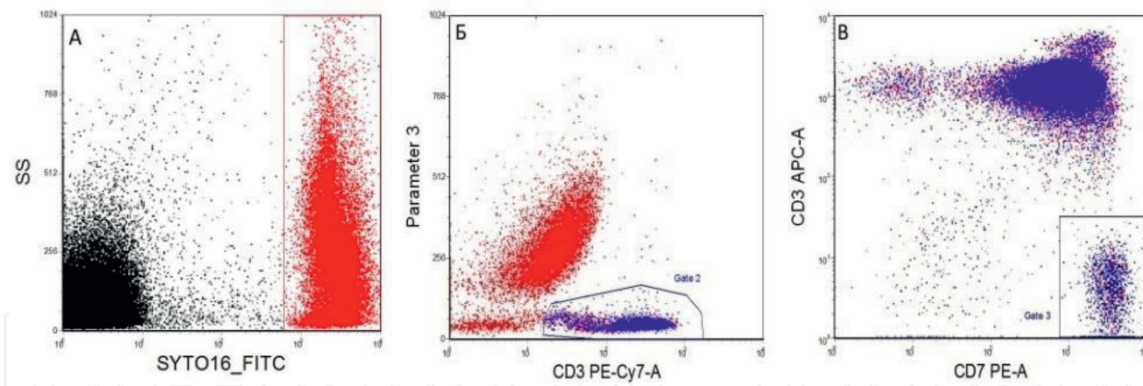


Figure 10.

An example of MRD-positivity with no blasts in the BM specimen by morphology on day 33 of therapy. Cytogram A: shows NC of the specimen (gate 1, red) basing on reactivity with Syto16 (X-axis) against SSC. Cytogram B: cyCD3⁺ T-cells within NC (gate 2, blue) with low SSC parameters (cyCD3, X-axis against SSC Y-axis). Cytogram C: CD7, X-axis against smCD3, Y-axis (gate 2) shows TCP (MRD cells) with CD3⁺CD7⁻ smCD3⁻ immunophenotype that are 2.69% of myelokaryocytes: MRD-positivity.

It is important to note that MRD negativity was not reported on day 15 of induction chemotherapy in any of the patients, that is, none of the patients achieved complete leukemic cytoreduction in the middle of remission induction therapy.

3.3.2 MRD assessment on day 33 of therapy

Quantification of residual blasts on day 33 of induction chemotherapy was made in 26 patients.

Most BM specimens (25/26) were included into group M1, while there was only one specimen meeting the M2 criteria. None of the specimens was included in group M3 basing on morphological characteristics.

We compared morphological and immunological findings. Specimens from the standard and intermediate risk groups were analyzed in detail with respect to MRD content.

All BM specimens from group M1 were MRD-positive. Basing on the immunological criteria, 16.0% of BM specimens (n = 4) were included in the standard risk group (MRD < 0.1%). The intermediate risk group consisted of 20 of 25 BM specimens (80.0%). One BM specimen from this group contained no blast cells by morphology, though FC discovered 2.69% of MRD cells (**Figure 10**).

One specimen was included in the high risk group by MRD assessment (MRD ≥ 10.0%).

Note that specimens from this sample demonstrated rather high lymphocytosis (more than 28.0%).

By morphological criteria, only one BM specimen was included in the M2 group (blasts 10.8%), while by immunological characteristics, this specimen might be referred to the standard risk group (MRD cells 0.04%).

CD7 in combination with CD3 are used to assess MRD in T-ALL in the BFM protocol. However, CD7 is commonly found on myelokaryocytes including both lymphoid and myeloid progenitors. Basing on ontogenetic characteristics of expression of T-lineage antigens, we propose to detect TCP (that is MRD cells) by expression of cytoplasmatic CD3 as the earliest lineage-specific T-cell marker.

Comparison of these two approaches to MRD monitoring (the standard BFM protocol basing on MRD cell count within CD7-positive cells and MRD count within cyCD3⁺ cells), both on days 15 and 33 of induction chemotherapy allowed us to make the conclusion that MRD quantification in T-ALL by detection of cyCD3⁺ smCD3⁻ populations is more accurate.

4. Conclusions and discussion

Successful treatment of patients with acute leukemia is associated with escalation of chemotherapy doses which requires effective monitoring of response. Assessment of response in key points of chemotherapy protocols helps both to stratify patients by risk groups more accurately and to avoid serious long-term side effects or patient overtreatment.

Search for adequate approaches to assessment of response was started since the 1970s. For a long time, response to therapy was assessed basing on the number of blasts in BM specimens as determined by morphological study, and as a result so called M-types were defined as follows: M1: <5.0% of blasts, M2: 5–25% of blasts, and M3: \geq 25% of blasts [22].

However, the progress in molecular methods such as polymerase chain reaction (PCR) and flow cytometry (FC) allowed quantification of minimal number of residual blasts at submicroscopic level (<0.01%).

In recent years, quantification of residual tumor cells in BM, that is, minimal residual disease (MRD) was a main criterion of response to chemotherapy. Opposite to myeloid leukemias when molecular genetic methods play the leading role in MRD detection, it is immunological approaches involving FC alone that can detect MRD cells in more than 95.0% of cases [23].

Both PCR and FC have their advantages and disadvantages. Implementation of multiparameter FC able to assess up to 12 parameters of a single cell and development of computer software that allows tens of millions of cells to be examined makes FC close to PCR by sensitivity [24]. FC is currently the most rapid and less costly approach to MRD monitoring.

In adults, problems of the most important detection points are a matter of discussion [2]. The MRD significance is most vivid in pediatrics. MRD is found to play a role both in ALL prognosis and recurrence prediction [1]. Key points of MRD monitoring and their clinical significance have been determined as well as MRD levels that allow detailed risk stratification of patients.

It is in childhood oncology that the MRD effects of the patient faith have been studied [1, 25]. The current protocols defined three mandatory time points such as the middle and end of remission induction chemotherapy (days 15 and 33) and the end of consolidation induction (day 78).

MRD monitoring plays different roles at different chemotherapy stages. The most important point in terms of the patient faith is the middle of induction chemotherapy when primary tumor response and prognosis are determined.

For a long time, there were multiple attempts to make the earliest possible assessment of response. One of the approaches is count of blasts in peripheral blood on day 8 of treatment. Patients with blast reduction below 1000 cells/mcl are a group of good response and good prognosis. However, the blast clearance on day 8 is found to reflect response to steroids only [5], while MRD level on day 15 of induction chemotherapy is the most accurate reflection of leukemic cytoreduction, that is, primary response to treatment [5, 14].

The key moment is the selection of good response patients (MRD < 0.01%) in whom reduction of anthracycline dose may be considered [11, 26]. In our study, FC discovered MRD-negativity in 11.5% of patients in whom reduction of anthracycline dose could be considered. These patients did not develop recurrence.

The situation in BM by day 15 of therapy is unique in terms of immunology. Since BCP are highly sensitive to glucocorticoids [17] that are the basis for treatment at the given stage, these cells are fully eliminated from BM [16]. This observation was a basis for a FC protocol developed by the St Jude Children's Research Hospital (Memphis, USA) study group for detection of MRD cells on day 15. This protocol

involves MRD monitoring within BM mononuclear fraction, while the BFM protocol uses recount for NC in the specimen.

We compared the two approaches to conclude that recount for NC (the St. Jude Hospital protocol) is the most robust criterion for risk stratification of patients. Moreover, analysis of MRD cell immunophenotype with respect to CD58 and CD38 expression demonstrated that the number of CD10⁺ B-cells was similar to that of CD58⁺⁺/CD38^{low/-} BCP in 36.4% of cases only. While the number of CD58⁺⁺CD38^{low} BCP was less as compared to CD10⁺ B-cells in the remaining specimens of aberrant BCP. We conclude therefore that quantification of MRD should be based on detection of BCP rather than of aberrant blasts.

MRD cells on day 15 are BCP. We used a variety of monoclonal antibodies for their monitoring. In pre-pre-B and pre-B ALL immunosubtypes, it is just enough to count CD34⁺ and/or CD10⁺ BCP. In case of pro-B immunosubtype (CD10⁻ and/or CD34⁻), MRD detection should involve a combination of nuclear TdT with cytoplasmic CD22 or CD79 α that show stable expression as demonstrated already in early studies of B-cell ontogenesis [27, 28].

Important properties of BM on day 15 are hypocellularity and a marked proportion of debris, that is, destroyed cells under the effect of systemic chemotherapy [29, 30]. This requires modification of FC protocols for MRD monitoring, namely use of special dies able to identify viable, not destroyed cells in specimens. Our study showed reasonable to use Syto nucleotropic dies that can clearly select NC and as a consequence help to avoid debris dilution that may result in a considerable underestimation of MRD count and interfere with risk stratification.

By the end of induction chemotherapy, appearance (regeneration) of normal B-lineage progenitors in BM makes MRD detection more difficult. Clear-cut distinguishing between MRD cells and regenerating BCP is possible basing on LAIP or aberrant immunophenotype.

Aberrance is currently defined as follows:

1. Different levels of Ag expressions (overexpression of some molecules [CD58, CD10, CD9], low or no expression of Ag such as CD38, CD81)
2. Expression of Ag of uncommon differentiation lineages (e.g., co-expression of myeloid Ag such as CD13, CD33, CD66c, CD123 on tumor blasts)
3. Asynchronous expression of lineage-specific Ag (expression of Ag of mature B-cells, e.g., CD20 on CD34⁺ BCP)

A combination of CD58 and CD38 is the most frequently used Ag combination for assessment of B-lymphoblast immunophenotype aberrance in the AIEOP-BFM and COG FC protocols.

Normal BCP are characterized by CD58^{+/low}/CD38^{+/++} immunophenotype, while aberrant immunophenotypes demonstrate CD58 overexpression and no or low CD38 expression.

As shown by the I-BFM-ALL-MRD Study group, CD58 overexpression is present on overwhelming majority of tumor B-lymphoblasts and is found in 93.5% of cases [31, 32, 19].

CD38 molecule with receptor and enzymatic activities that is involved in B- and T-cell ontogenesis is brightly expressed on normal BM progenitor cells. Low CD38 expression in BCP is considered aberrant [19, 41] and is found in 61.2% of B-ALL according to Min Xia et al.

In our study, CD58 was overexpressed in 71.7% of B-ALL cases, that is, less frequently than by literature data. An explanation may be that the boundary between

Ag normal and overexpression may be unclear. To make a more accurate evaluation of aberrant immunophenotype in primary diagnosis, Ag tumor expression should be compared with the number of residual normal BCP that is not always large. To make visualization more determinant, one has to select a considerable amount of cells (≥ 1 million), which was not possible in early studies.

There were only 58.6% of ALL with aberrant (low) CD38 expression which was in accord with foreign data.

If one of two Ag is not aberrant, MRD may be assessed basing on alternative combinations such as CD58/CD10, CD58/CD34, CD38/CD10, and CD38/CD34.

Of note, in our study, about one fourth of all cases (24.0%) had tumor B-lymphoblasts with no aberrance by this Ag combination. Therefore, additional criteria to identify aberrant immunophenotype are needed.

This requires MRD criteria to be used at time of diagnosis and most informative MRD markers to be selected in every specific case at the stage of ALL primary diagnosis.

Multicolor FC that simultaneously evaluates up to 12 various cell characteristics makes the most complete characterization of LAIP. This approach may be implemented by use of the EuroFlow consortium 8-color panels with optimal combination of stable markers and fluorochromes [33–35].

The panel for more accurate B-ALL diagnosis includes 3 basic (repeated in all specimens) Ag and 20 specific Ag. This helps to make multiple aberrance evaluation by expression of nonlineage-specific CD45, CD58, CD38, CD9, CD123, and CD81; by expression of noncommon differentiation lineages such as pan-myeloid CD13, CD33, CD66c, CD15, CD65; and by asynchronous expression of Ag of different stages of B-cell differentiation CD10, CD34, CD, CD22, CD20, CD21, CD24. It is of much importance that expression of all above-mentioned markers may be assessed within a single cell population, that is, $CD19^+CD34^+CD45^{low}$ BCP.

The protocol makes possible indirect prediction of clinically significant abnormalities basing on a proper immunophenotype [36]. For instance, immunophenotypes of cells with mutations BCR-ABL, MLL, Tel-AML-1, and E2APBX1 are described.

When analyzing capacities of EuroFlow 8-color protocols with respect to MRD monitoring, we paid attention to CD81 [35]. The CD81 molecule belongs to the tetraspanin family and is directly associated with CD19, thus forming a signal complex CD19-CD21-CD81 that realizes its functional activity in normal B-cell ontogenesis. By the literature [37], aberrant (weak) CD81 expression is found in 82% of B-ALL cases. Our evaluation of CD81 expression in primary diagnosis coincided with international study data. For instance, aberrant expression was found in 85.7% of primarily diagnosed ALL.

Another molecule from the tetraspanin family is of interest as a criterion for MRD detection, that is, CD9 [38–40]. Its expression is evaluated both in 4- and 6-color FC protocols of the COG study group (M. Borowitz). CD9 is brightly expressed on early BCP, disappears at the pre-B stage, and appears again on mature B-cells. Its monomorphic overexpression on BCP is considered aberrant. In our study, 87.5% of B-ALL cases demonstrated CD9 aberrant expression on tumor B-lymphoblasts.

In case of monomorphic co-expression of myeloid Ag such as CD13, CD33 and CD66c [31, 41] and also CD123 [42], they may be used as LAIP of tumor B-lymphoblasts. Prognostic significance of myeloid Ag co-expression on tumor cells is disputable, though CD66c (KORSA) is shown to be associated with BCR/ABL1 reconstructions [43, 44]. N. Guillaume et al. analyzed immunophenotype of ALL from BCP to discover CD66c co-expression to be the most frequent (40.0%), while pan-myeloid Ag CD13 and CD33 were detected in 15.0% of cases only.

In our evaluation of primary immunophenotype of tumor B-lymphoblasts, CD66c co-expression was found in 75.0% of cases, while CD13 and CD33 were present in 46.0%, which is similar to international study data.

As concerns interleukin-3 receptor α -chain (CD123), all B-ALL were aberrant (i.e., the Ag was expressed on tumor cells), which is in accord with L. Munoz et al. [45].

Asynchronous expression of Ag of noncharacteristic differentiation lineages is an additional criterion for tumor B-lymphocyte LAIP. CD20 is such a marker in CD20-positive B-ALL whose expression is characteristic of mature B-cells only. However, CD20 co-expression on tumor B-lymphoblasts is found in 6.4–15.3% of B-ALL [29, 30]. CD20 is included in most FC panels for MRD diagnosis though it is appropriate rather for more clear-cut identification of CD20⁻ BCP than for evaluation of its aberrant expression. This finding may be explained by a small cohort of B-ALL patients (up to 16.0% of all B-ALL patients) in whom residual blasts may be identified on the basis of CD34⁺CD20⁻ immunophenotype. In our study, only 14.7% of ALL patients demonstrated CD20 co-expression on tumor B-lymphoblasts.

So in terms of FC approaches to evaluation of small populations which may also include MRD determination of LAIP is most effective using a combination of two Ag with aberrance manifesting itself as overexpression or a combination of molecules with over- and low/no expression. We propose the following Ag combinations: CD123⁺CD81^{low}, and CD9⁺⁺CD81^{low}.

Remission completeness is clinically assessed on day 33 of treatment. In our study, MRD-negative status was detected in 60.0% of patients.

Most patients (65.0%) receiving treatment by ALL IC BFM 2009 schedule were stratified into the intermediate risk group by MRD content.

Day 78 of treatment (end of consolidation induction) is the third time point with undoubted prognostic value for MRD detection. Basing on this evaluation, a group of so called slow response may be identified basing on MRD status. These patients are MRD-positive on days 15 and 33 and reach MRD negativity by day 78 only. This cohort is characterized by good prognosis.

In our study, 64.3% of patients were MRD negative, with 7 of 14 patients followed-up in all the three points belonging to the slow response group.

Tumor immunophenotyping in disease recurrence is of special interest. Changes in Ag expression during treatment are studied and described rather well. According to international study data, changes as compared to disease onset may occur also in tumor cell immunophenotype in disease recurrence.

We assessed blast immunophenotype both in disease onset and relapse in five patients. Tumor blast immunophenotype was the same by the main diagnostic Ag in disease onset and recurrence in four of the five patients. Of interest is that change in blast phenotype as concerns intensity of CD58/CD38 expression versus the primary diagnosis was found in two of the five patients.

Clinical value of immunophenotype change as concern aberrance markers needs further clarification and may become an additional criterion in the protocol.

Novel targeted therapies that influence disease at molecular level are implemented in clinical practice every year and many are at various stages of clinical trial.

A new stage of progress in targeted therapy is associated with development of biospecific antibodies that are a new class of monoclonal antibodies (MAb) binding with a surface antigen target, on the one hand, and with T-cell receptor, on the other hand, thus recruiting effector T-cells and enhancing tumor response. Blinatumomab (Blinzito) is the first therapy in this class that is approved by FDA for the treatment of Ph-negative refractory/relapse ALL from B-LP [38]. This approval was based on results of phase II clinical trials with 43% of 189 adults achieving complete response and 82% achieving MRD-negativity [46].

Notwithstanding initial treatment results, some patients fail to respond to blinatumomab or develop progressive disease after initial response. Recurrence rate is 30%.

Characterization of blast immunophenotype in recurrence on blinatumomab therapy discovered no CD19 expression on tumor cells. This interferes with BCP identification and requires new methodological approaches to MRD assessment.

Inclusion of blinatumomab into B-ALL therapy requires an alternative MRD evaluation strategy.

There was one patient receiving blinatumomab in our study [47]. The MRD monitoring and immunophenotyping at second recurrence was difficult due to the absence of CD19⁺ B-cells. An algorithm for BCP detection was proposed and tested that was based on alternative B-lineage differentiation markers. Nuclear nuTdT in combination with cytoplasmatic CD22 as most stably expressed Ag were chosen.

So, given the appearance of new targeted therapies, FC algorithms for both MRD diagnosis and monitoring require certain flexibility and timely rational changes.

There is an equivocal situation with T-ALL. On the one hand, search for aberrant immunophenotype for TCP ended in failure. Expression of main Ag studied with respect to LAIP, that is, CD99 and nuTdT is variable [21]. On the other hand, taking into account normal T-cell ontogenesis, MRD assessment may be based on the absence of TCP of the cyCD3⁺⁺smCD3⁻nuTdT⁺CD7⁺ level in BM. One has therefore just to assess TCP number in order to quantify MRD at any therapy stage.

Correct choice of antibody CD3 clone is of much importance for TCP identification. For instance, clone UCHT1 should be used to detect CD3 cytoplasmatic expression, while CD7 is needed to detect the membrane determinant.

The AIEOP-BFM protocol suggests that MRD detection in T-ALL should be based on CD7.

We compared the two approaches to TCP identification to find optimal TCP detection with CD7^{+/++}smCD3⁺ immunophenotype within cyCD3⁺ population basing on T-cell ontogenesis.

All T-ALL patients in our study were stratified into the intermediate and high risk groups. According to MRD levels on day 15, most patients remained in the initial stratification group though 25.0% were transferred from the intermediate into the high risk group.

Of note that all patients demonstrated MRD-positivity on day 15, that is, none of the T-ALL patients achieved complete leukemic cytoreduction on ALL-IC-BFM 2009 therapy.

So, clinical significance of MRD in ALL arises no doubt. It is reasonable to make MRD quantification immunologically using FC assay.

Since there are no normal BCP on day 15 of induction chemotherapy, MRD quantification in case of pre-pre-B and pre-B ALL immunosubtypes should be based on detection of CD10⁺/CD34⁺ population within CD19⁺ B-cells. In case of pro-B (CD10⁻ and/or CD34⁻), ALL immunosubtype MRD detection should be based on expression of nuclear nuTdT in combination with cyCD22.

During BM regeneration (end of remission induction therapy and long-term treatment stages), MRD quantification is based on identification of BCP with aberrant immunosubtype. Alongside with the most common CD58/CD38, the following Ag should be used in LAIP assessment: CD81, CD9, CD13, CD33, CD66c, CD123, and CD20. At the stage of diagnosis, the most informative personalized Ag combinations should be selected for further MRD monitoring to make a more accurate risk stratification of patients at different therapy stages and are an effective tool for its modification.

Tumor immunophenotype in disease recurrence should be compared with that in disease onset taking into account its possible changes.

In case of targeted therapy, for example, anti-recurrence treatment, FC protocol should be changed with respect to treatment features. For instance, BCP evaluation

on blinatumomab therapy should be based on expression of nuTdt in combination with cyCD22.

In T-ALL, MRD assessment at any therapy stage may be limited to quantification of CD7^{+/+}smCD3⁻ cells within cyCD3⁺ population.

Conflict of interest

The authors declare no conflict of interests.

Appendixes and nomenclature

Ab	antibody
Ag	antigene
ALOT	acute leukemia orientation tube
ALL	acute lymphoblastic leukemia
ALL-IC-BFM	intercontinental acute lymphoblastic leukemia protocol of Berlin-Frankfurt-Munster study group
B-ALL	ALL from B-cell precursors
BCP	B-cell precursors
BFM	Berlin-Frankfurt-Munster study group
BM	bone marrow
CD	cluster of differentiation
cyCD	cytoplasmic cluster of differentiation
COG	children oncology group
FC	flow cytometry
LAIP	leukemia-associated immunophenotype
MAb	monoclonal antibodies
MRD	minimal residual disease
MNC	mononuclear cells
NC	nucleated cells
smCD	membrane cluster of differentiation
T-ALL	ALL from T-cell precursors
TCP	T-cell precursors

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