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Flow-cytometric MRD detection in pediatric T-ALL: a multicenter AIEOP-BFM consensus-based guided standardized approach

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Abstract

Objectives: Risk-based stratification approaches using measurable residual disease (MRD) successfully help to identify T-acute lymphoblastic leukemia (T-ALL) patients at risk of relapse, whose treatment outcomes are very poor. Because of T-ALL heterogeneity and rarity, a reliable and standardized approach for flow cytometry (FC)-based MRD measurement and analysis is often missing.

Methods: Within the international AIEOP-BFM-ALL-FLOW study group we made a consensus on markers and a standard operating procedure for common 8- and 12-color T-ALL MRD panels. Custom manufactured tubes with dried backbone antibodies were tested in parallel to local FC standards.

Results: Altogether, 66 diagnostic and 67 day 15 samples were analyzed. We designed two guided MRD gating strategies to identify blast cells in parallel to expert-based evaluation. We proved that the optimized tubes allowed the correct identification of blast cells in all diagnostic samples. Both, expert and guided analysis of day 15 samples correlated to local standard (Spearman R=0.98 and R=0.94, respectively). Only in 2 (3 %) and 4 (6 %) patients expert gating and guided analysis results were substantially discordant from local standard, respectively. The cases that require an individualized approach may be partially identified at diagnosis through a rare immunophenotype or mixed phenotype acute leukemia status.

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Conclusions: Our work shows that standardized operating procedures together with guided analysis are applicable in a great majority of T-ALL cases. Further improvement of MRD detection is needed, as in some cases an individualized analytical approach is still required due to the challenging nature of the T-ALL phenotype.

Keywords: flow cytometry; standardization; minimal residual disease; T acute lymphoblastic leukemia

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) comprises about 15 % of all newly diagnosed ALL cases in pediatric patients [1]. The 5-year event-free survival rate exceeds 80 % [2, 3] and the overall survival (OS) rate 90 %. Nevertheless, the prognosis for relapsed patients remains very poor with 5-year OS<25 % [1]. Risk stratification relies mainly on response to treatment (i.e. Prednisone response and minimal/measurable residual disease, MRD) [2–4]. Both molecular and multicolor flow cytometry (FC)-based approaches are used for MRD assessment in the T-ALL clinical setting and risk stratification. The success rate of detecting appropriate immunoglobulin (IG) and T-cell receptor (TR) rearrangements as MRD targets is lower in T-ALL compared to patients with B cell precursor (BCP) ALL [5, 6].

For this reason, FC-based MRD detection represents a decisive tool in this setting. However, immunophenotypic blast identification could be challenging in some cases; T-ALL could show a variety of phenotypes [7], and modulation of expressed markers was also described during the treatment [8, 9]. Therefore, several panels were described in the literature (e.g. in references [7–14]), mostly with an overlapping backbone of CD7, CD5, CD3, CD45 and varying use of additional markers, such as CD99, nuclear (nu)TDT, cytoplasmic CD3 (cyCD3), CD4, CD8, CD34, CD2, CD48. Standardized approaches for both, the pre-analytical and analytical phases are still lacking. Due to the rarity of the disease, building expertise is a challenge for less trained or small laboratories, so there is a need for a more standardized approach allowing more accurate T-ALL MRD measurement in routine practice and identification of cases that require special expert attention.

Within the collaborative group of Associazone Italiana Ematologia Oncologia Pediatrica Berlin-Frankfurt-Münster ALL Flow laboratories (AIEOP-BFM-ALL-FLOW), we aimed to develop and validate a consensus panel to be incorporated into the routine of reference laboratories. Due to different cytometer configurations, we evaluated both 8-color and 12-color versions of antibody panels. These panels and respective gating strategies were tested prospectively and the results were compared to local standard 8-10-color FC T-ALL MRD panels.

Materials and methods

Between 10/2018 and 01/2023 we collected data from 66 diagnostic samples (peripheral blood (PB) or bone marrow (BM)) and 67 day 15 (d15) BM samples from a total of 81 pediatric patients diagnosed with T-ALL/T-lymphoblastic lymphoma with leukemic presentation among eight experienced centers belonging to the AIEOP–BFM-ALL-FLOW study group. The sample details are shown in Supplementary Table S1. Informed consent was obtained from all patients and their guardians in accordance with the Declaration of Helsinki. Residual amount of samples not used for routine diagnostics was used for testing. The 8-color and 12-color versions of the antibody panels were developed sharing the same dried backbone, manufactured by Exbio company (Vestec, Czechia). The panels were established as a result of a validation process that began with a detailed survey among the participating centers (Supplementary Figure S1). The survey was based on the selection of markers with higher levels of informativeness in the T-ALL MRD diagnostic routine. The marker composition of the final version of the panels is shown in Table 1, and the detailed antibody composition is described in Supplementary Table S2. Due to the limited configuration, we could not include CD1a and CD34 in the 8-color panel, as real-life experience has shown them to be less informative than others. The majority of samples (n=87) were measured using the original version of the panel, while 46 samples were measured with the updated version, with fluorochrome change in CD3 and CD8 antibodies.

To assess background staining in non-T-ALL samples, control samples (non-malignant PB samples (n=19), non-malignant BM samples (n=9) and samples from patients with BCP-ALL on d15 of induction treatment (n=11) were measured using the original version of the panel.

Samples were processed according to current standard operating procedure (SOP). The first version of the SOP included red blood cell lysis with Excellyse (Exbio, Vestec, Czechia; n=92), while the updated version switched to BD FACS Lysing solution (Franklin Lakes, New Jersey, USA; n=42). One diagnostic sample was processed in parallel according to both SOPs. Intracellular staining was performed using FIX&PERM® Cell Fixation and Permeabilization Kit (Nordic Mubio, Susteren, The Netherlands) (Supplementary Methods). The final version of the SOP is included in Supplemental data. Data were analyzed using FlowJo software (BD, Franklin Lakes, NJ, USA). Spearman test was used for correlation analysis if not stated otherwise. Statistics was performed in GraphPad (San Diego, CA, USA). Molecular

Table 1: Final version of the panels.

AIEOP-BFM-ALL-FLOW T-All MRD 12 colors tubes panel												
Tube	**Pacific Blue**	**Pacific Orange**	**FITC**	**PE**	**PerCP-Cy5.5**	**PE-Cy7**	**APC**	**AF700**	**APC-Cy7**	**BV605**	**BV711**	**BV786**
Tube #1	Syto41	**CD45**	**CD99**	CD48	**CD8**	**CD5**	**CD7**	CD16 +CD56	**CD3**	CD1a	CD4	CD34
Tube #2	cyCD3	**CD45**	**CD99**	CD48	**CD8**	**CD5**	**CD7**	CD16 +CD56	**CD3**		CD4	CD34

AIEOP-BFM-ALL-Flow T-ALL MRD 8 colors tubes panel								
Tube	**Pacific Blue**	**Pacific Orange**	**FITC**	**PE**	**PerCP-Cy5.5**	**PE-Cy7**	**APC**	**APC-Cy7**
Tube #1	Syto 41	**CD45**	**CD99**	CD48	**CD8**	**CD5**	**CD7**	**CD3**
Tube #2	CD4	**CD45**	**CD99**	CD16 + CD56	**CD8**	**CD5**	**CD7**	**CD3**
Tube #3	cyCD3	**CD45**	**CD99**	CD34	**CD8**	**CD5**	**CD7**	**CD3**

In bold, dried backbone is marked. In the original version, the position of CD3 and CD8 was swapped. Cy cytoplasmic, AF Alexa Fluor; BV Brilliant Violet.

MRD was investigated using IG/TR real-time quantitative PCR in different centers (n=50) following current EuroMRD protocols as described elsewhere [15, 16].

Results

Antibody panel development and protocol optimization

A consensus was made regarding sample preparation, antibody choice and acquisition. Due to different cytometer configurations in the participating centers, both 8-color (containing 3 tubes) and 12-color (containing 2 tubes) panels were developed. A common set of antibodies among both panels was used in dry format (Table 1). We tested different conditions (Excellyse vs. BD FACS Lyse) and changed the antibody format during the study (originally CD8 APC-Cy7 and CD3 PerCP-Cy5.5, then CD3 APC-Cy7 and CD8 PerCP-Cy5.5) (Online Supplementary Methods and Supplementary Figures S2-S3). Samples processed with all variants are included in subsequent analyses.

The developed T-ALL MRD panels are suitable for both, expert-based and gating strategy-guided identification of blasts

First, we tested whether the T-ALL MRD panels allowed identification of atypical blast cells in diagnostic samples. In total 66 diagnostic samples were stained. Of these, we performed centralized expert gating by one of the two experts ("expert-based gating") and compared the % of blast with those from local analysis ("local standard") (Supplementary Table S3). Additionally, we introduced two gating strategies ("guided gating strategy") targeting phenotypically different T-ALL stages: CD3negative (gating 1) and CD3positive blasts (gating 2), which we applied to all measured tubes #1–3 of the 8-color panel (tube 1 n=16; tube 2 n=15; tube 3 n=10) and to tube #1 of the 12-color panel (n=50) (Figure 1, Figures S4). Expert gating was based on a combination of leukemia-associated immunophenotyping (LAIP) and the different-from-normal (DfN) approach. In guided gating, the DfN approach was more dominant, although it still reflected the initial information about CD3, CD5, CD4, and CD8 expression as LAIP. For the 8-color panel, the higher of the expert blast values from the surface tube 1 or 2 was selected, with the surface tubes considered as standard. Gating 1 was the better fitting strategy (giving the higher number of blasts) in 65 % of cases and gating 2 in 35 % of cases (Supplementary Table S1).

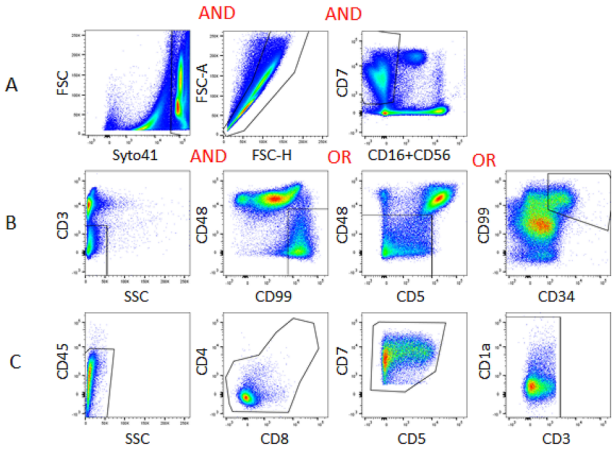

Figure 1: Gating strategy 1 for surface tube 1 of the 12-color panel is shown. As a first step (A), nucleated cells, excluding doublets and NK cells, are gated. (B) Next, aberrant phenotypes are identified: low CD48 or CD5, as well as high CD99 and CD34 levels. CD3 negativity was mandatory for gating 1 (CD3negative), while for gating 2 (CD3positive) CD3 positive events were selected. (C) In the final step, refinement and/or cluster gating was performed, incorporating information about initial CD5, CD1a, CD4 and/or CD8 expression, if available. The MRD percentage was calculated as MRD events out of all nucleated single cells ×100 %.

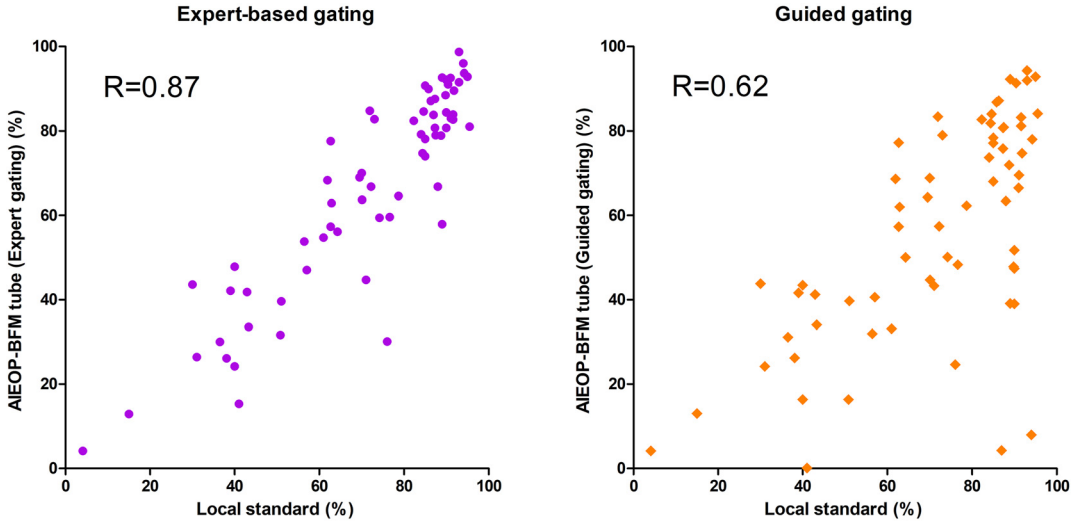

Figure 2: Flow cytometric analysis of T-ALL samples at diagnosis (n=66). On x axis local standard results (expressed value in % out of all nucleated cells), on y axis AIEOP-BFM tube results are shown. Left panel: expert approach (the highest result from tube #1-#2 in the 8-color panel or result from tube #1 in the 12-color panel), right panel: best guided gating strategy for tube #1-#3 with highest result of 8-color panel and tube #1 of 12-color panel. R values for the spearman test are shown (both p<0.0001).

As shown in Figure 2, both expert-based and best fitting guided gating strategy of the best tube (in case of 8-color panel), showed good correlation with the local standard data with a correlation coefficient of 0.87 and 0.62 (p<0.0001), respectively, indicating that the composition of the tubes is informative for blast identification. When blast values from guided analysis were compared to expert-based values we observed an underestimation in four samples by more than 2-fold (Supplementary Figure S5). This result could be explained by some peculiarities present in these samples such as an expression of CD56 (n=1), low CD7 expression (n=2) or partial CD3 expression (n=1). In conclusion, the antibody composition of tubes #1, 2 and 3 of the 8-color panel and tube #1 of the 12-color panel together with one of the guided gating strategies is capable of identifying blasts in 94 % of samples. For the remaining 6 % of samples expert gating was required to avoid missing relevant proportions of blast cells, due to the unexpected expression of core antigens.

Standardized pre-analytical procedures and guided gating strategies allow for reliable FC-based MRD detection and correlates well to PCR MRD

To validate the developed guided gating strategy approach, we applied both the expert and the guided analysis on 67 d15 T-ALL samples. The best guided gating strategy fitting at diagnosis was chosen for d15 samples, unless the immunophenotype changed significantly (n=6). The highest result of the measured tubes of the 8-color panel (tube #1 n=15; tube #2 n=15; tube #3 n=8) was chosen for statistical evaluation (Supplementary Table S1). In all samples at least 50,000 nucleated cells were measured (median 474 376 nucleated cells, range 51 739 - 1 192 501). A minimum of 10 blast events were needed to report the MRD value, otherwise the sample was reported as negative [17, 18].

Both gating approaches demonstrated a very good correlation (0.98 and 0.95, p<0.0001) to the reported local standard MRD values (Figure 3). With respect to risk stratification cut-offs used in current pediatric ALL treatment protocols (i.e. 0.1 and 10 %), 62 out of 67 (93 %) of the expert values and 60 out of 67 (90 %) of the guided gating values were concordant with the local standard. Due to the unusual antigen expression (such as high CD48 expression, positive CD56, low CD7 and/or CD99 expression) the guided analysis missed the MRD population in two d15 samples, and expert-based gating was necessary. In five samples both the guided and the expert analysis did not correlate with the local standard values in terms of risk stratification: In 3/5 (UPN22, UPN29 and UPN58), the local MRD values were 9.5 , 10.3 and 11.9 % (compared to the expert values 16.8 , 6.7 and 5.8 %, Supplementary Table S1) and thus borderline for classification. One patient was classified as bilineal (UPN48) and the T-MRD analysis missed the myeloid subpopulation. Only in one patient (UPN63) with a local MRD of 23.4 % and expert MRD of 8.1 % the classification would have been significantly different between expert/guided gating results and local standard value, with

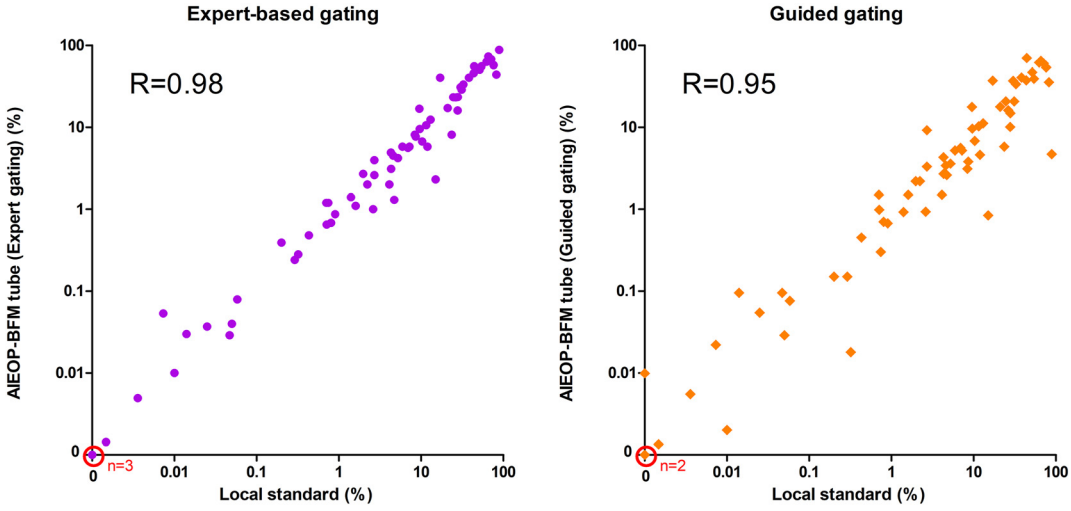

Figure 3: Flow cytometric analysis of T-ALL samples at d15 (n=67). On the x axis local standard results (expressed value in % out of all nucleated cells), on y axis AIEOP-BFM tube results are shown. Left panel: expert approach (the highest result from tube #1-#2 in the 8-color panel or result from tube #1 in the 12-color panel), right panel: best guided gating strategy for tube #1-#3 of 8-color panel with highest result and tube #1 of 12-color panel. R values for the spearman test are shown (both p<0.0001).

'intermediate risk' instead of 'high risk', respectively. Except for a possible variability in sample preparation (although being measured the same day) a clear explanation for this case could not be identified. Two of the four discrepant cases, which were not considered as borderline, could have been identified at diagnosis due to a rare immunophenotype or mixed phenotype acute leukemia status. Next, we compared the FC-MRD data with PCR MRD results. The correlation was good when using both the expert and guided gating strategies (R=0.82 and R=0.80, respectively), equal to correlation with local standard values (R=0.79) (Supplementary Figure S6).

In the analysis of MRD data, the twelve-color (one surface tube) and eight-color (two surface tubes) panels performed similarly (Supplementary Figure S7).

In conclusion, MRD values from both the expert and the guided analysis led to concordant risk classification in the vast majority of cases proving that the proposed standardized pre-analytical (SOP and panel design) and analytical strategies are comparable to local standard of experienced laboratories and allow the advantage of a common and easily comparable approach within the AIEOP-BFM consortium.

Background staining in control samples reveals an acceptable specificity of the analysis

To investigate the staining- and analytical-based background of our procedures, we applied both guided gating strategies on different types of control samples such as BM from patients with BCP-ALL on d15 of treatment or BM/PB from patients without hematological malignancies (Online Supplementary Methods). To this purpose we calculated in how many control samples the background was below 0.1 % (Supplementary Figure S8). We observed that MRD gating of surface CD3 positive blast cells was the most challenging, as these can significantly overlap with non-malignant T cells. Background for surface CD3 negative immunophenotype (gating 1) was acceptable in more than 90 % of all measured control samples with tube #1 or #2, while tube #3 of the 8-color panel showed poorer performance, which was due to the lack of specific markers for precise gating in this tube. However it should be noted that the primary objective of this tube was mainly to explore the utility of cytoplasmic CD3 staining.

Utility of cytoplasmic CD3 staining for the developed gating strategies on d15 T-ALL and control samples

Finally, we sought to determine whether cyCD3 staining improved T-ALL MRD identification in the tested tubes. We included a cyCD3 gate at the end of the best fitting gating strategy in tube #3 of 8-color and tube #2 of the 12-color panel and compared the results with the local standard analysis (n=40; Figure 4). The correlation with the local standard analysis was R=0.98 and R=0.95 (p<0.0001) without and with cyCD3 gating, respectively. In only one case would the MRD level change between the categories 0–0.1 %, 0.1–10 % and 10–100 % if cyCD3 gating step was added because of the very low cyCD3 expression (case UPN53). Interestingly, in 7 out of 38 MRD-positive samples with cyCD3 data, only 50 % or less blast

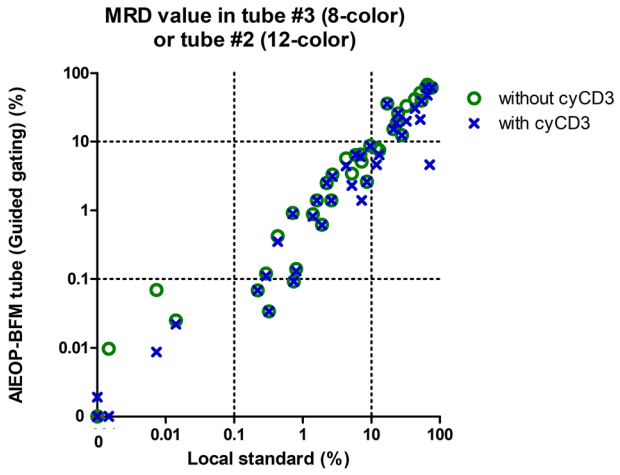

Figure 4: Comparison of results of applied guided gating strategy with and without cytoplasmic CD3 (cyCD3) of tube #2 of 12-color panel and tube #3 of 8-color panel (n=40). On the x axis local standard result (expressed value in % out of all nucleated cells), on the y axis the AIEOP-BFM tube results are shown. R values for the spearman test were 0.98 and 0.95 for analysis without and with cyCD3, respectively (both p<0.0001).

cells (assessed by expert gating) expressed cyCD3. Applying the cyCD3 gate would then lead to at least a 2-fold underestimation of MRD value.

Next, we applied cyCD3 gating on the results of the guided analysis of control samples (Supplementary Figure S8). We observed a slight improvement of background for gating 1 but no value for gating 2.

We conclude that cyCD3 staining on d15 is neither useful for MRD gating nor for increasing specificity.

Discussion

Our study aimed to explore a consensus-based approach for T-ALL MRD analysis in a BFM-oriented multicenter setting based on the best current knowledge in the field, providing consistent data for use in patient risk stratification and clinical-biological correlation studies.

As part of this process, we aimed to provide a consensus antibody panel, a robust sample-preparation SOP, and a guided gating analytical strategy to assist in the otherwise mostly expert based approach of gating T-ALL MRD.

By analyzing diagnostic and d15 T-ALL samples, we demonstrated that the proposed panel composition enables the identification of blast cells by both the expert- and the guided-gating strategies. In the majority of cases where the results of the guided analysis were discordant, the expert gating was also discordant with the local standard analysis. In two cases where the guided gating strategy approach failed (but the expert did not), the phenotypes that impeded the analysis were identified, such as (partial) negativity of CD7, high expression of CD48, low expression of CD99 or positivity of CD56. We included CD48 and CD99 as the most important parameters for the guided MRD gating, in accordance with a recent publication by Kowarsch et al. [7] In our 8-color panel, only tube #1 contained both these markers and therefore most of the participating laboratories in this study prioritized this tube when a limited amount of material was available. Of note, the discordant results of the expert analysis were found mostly in MRD values around the cut-off of 10 % and originate from the variability in FC measurement. In one of the discordant samples, we observed a difference in MRD values between surface and intracellular staining, highlighting the importance of the apoptotic blast population and the variability arising from the type of sample processing.

Tembhare et al. [19], introduced 10-color tubes with 11-markers for T-ALL-MRD, CD5 vs. CD38 expression was used to differentiate between T cells, NK cells, myeloid progenitors, plasmacytoid dendritic cells. The blast cells with one of Leukemia-associated immunophenotypes (LAIP) were identified using CD38, CD4 vs. CD8, CD5 or CD34. In cases with surface CD3 expression, the MRD identification was based on the expression pattern of CD3 and CD4 vs. CD8. However, the authors based their approach mostly on the dissection of immunophenotypically different cell subpopulations rather than on the straightforward gating leading to the assignment of a final “blast population”. In our study we used the latter approach and showed that it led to a high degree of concordance between the guided gating and the expert gating strategy (with only 3 % of cases that would be classified differently by the two gating approaches used on the same tube).

When evaluating the antibody panel, attention must be paid to the compensation issues that may affect the final analytical results. In our panel, this involved the spillover from CD99-FITC to CD48-PE and from CD99-FITC to CD3-PerCP-Cy5.5. The latter eventually led to a change in the panel, to allow better assessment of CD3 positivity. Since CD3 expression was crucial in deciding which of the two guided gating strategies to use (as was also done by other groups, e.g. Singh et al. [12]), differentiation of those cells in case of spillover issues and/or heterogeneous expression or its modulation requires expert based gating. To minimize such issues, we introduced tubes with dried antibodies, which have become the current standard in FC and provide fluorochrome stability, comparable staining patterns and thus uniform spillover values in one batch of produced tubes. Within our collaborative network, we were able to share these custom-made tubes and thus meet the requirement for a minimum production batch size despite the small number of samples due to the rarity of the disease.

Cytoplasmic staining of CD3 is considered a milestone in several published T-ALL FC panels. We included this marker in one of the tubes of both the 8-color and 12-color panels. However, we were unable to demonstrate additional value of cyCD3 for MRD gating at d15 of therapy. We did not encounter any difficulties in gating immature T-ALL subtypes (T-I or T-II, including early T cell progenitor, ETP) without the use of cyCD3, thus highlighting the added value of CD99 and CD48, and NK-cells could be well distinguished using other markers present (CD5, CD16, CD56, surface CD3) [20]. Of note, there were 13 ETP cases with measured d15 samples in our cohort. We did not observe a higher proportion of discordant MRD values (evaluated using surface tubes) from either expert or guided gating compared to the local standard. Furthermore, cyCD3 is not helpful in distinguishing blasts from mature T cells in mature T-ALL (T-IV). Finally, we observed partial cyCD3 expression in some atypical blasts (18 % of cases had 50 % or less cyCD3 expression). Thus, if intracellular TdT staining, which was shown to be significantly downregulated during the treatment [8], is omitted, permeabilizing staining procedures required to stain cyCD3 are questionable at least in MRD settings such as d15 where high levels of MRD are present.

In this view, the position of cyCD3, as well as of other poorly informative markers such as CD34 or CD1a (data not shown), could potentially be replaced by other markers such as CD38 for exclusion of NK cells as well as targetable antigen, CD2, nuTDT, HLA-DR, CD45RA or myeloid markers (CD117, CD13, CD33) as used by other groups [11, 12].

In our work, we introduced two gating strategies to classical four-stages classification of T-ALL. With respect to immunophenotyping, subtypes pro-T (T-I), pre-T (T-II) and part of intermediate (T-III) are CD3-negative and the rest of T-III and mature (T-IV) cases are CD3-positive. This influences the immunophenotyping strategy more than the actual EGIL stage, as was used by other groups as well [12, 19].

Our data highlight that surface CD3 positive T-ALL cases remain a challenge as the non-malignant background for such cells is higher than the cut-offs commonly used in risk stratification. In 28 % of MRD positive samples in our cohort, CD3 was expressed on at least 50 % of the blasts. These samples were therefore analyzed using gating strategy 2. Interestingly, there was no discordant MRD result with local analysis, suggesting that the use of all markers together and/or the knowledge of the initial immunophenotype may provide enough information to reliably interpret the data. This concept also emerges in the study of by Tembhare et al. [19] highlighting the importance of CD4 and CD8 expression along with CD3 intensity in the CD3 positive T-ALL subtype. Indeed, both these markers were included in our panel.

In conclusion, based on the present experience, the best laboratory routine should include measurement of either tube #1 of the 12-color panel or tubes #1 + 2 of the 8-color panel, followed by the application of guided gating 1 or 2, depending on the surface CD3 expression at diagnosis. If the expression is heterogeneous, both gating strategies should be used to identify the blast cells. The study was conducted during the transition from the 8-color standard to the 12-color standard in clinical cytometry, and we expect that 12 color instruments will become a standard in the near future.

We cannot exclude the possibility that in the future novel markers and combinations may improve T-ALL MRD assessment within particular minor sub-classes of T-ALL (e.g. ETP-ALL or ambiguous myeloid/T-ALL). In this regard, one limitation of our study may be the sole inclusion of d15 samples, which often contains a higher proportion of blast cells as compared to what is expected in most MRD samples at later time-points when higher sensitivity is required. Indeed, d15 MRD is currently the only FC-based time point relevant for patient stratification in the AIEOP-BFM ALL front line protocols except for rare patients without IG/TR targets. The aim of the study was to evaluate the benefit of standardization in the d15 context, where only nine cases with detectable MRD<0.1 % were identified. Caution should be exercised when extrapolating our findings to later time points with lower MRD involvement.

In summary, our results demonstrate the high informative value of the selected tubes, the robustness of the sample preparation procedure and the validity of the proposed guided gating strategies for a reliable and reproducible d15 MRD measurement in BFM-treated T-ALL patients.

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Research ethics: Ethical Committee approval 06/2019 (NU20-05-00282). All procedures were performed in accordance with the current revision of the Helsinki Declaration.

Informed consent: Informed consent was obtained from all individuals included in this study, or their legal guardians or wards.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission. All centers measured FC data and provided local tube analysis. MR and TK developed and optimized the panel, all centers provided feedback on final panel setup. TK and GG have designed the research. MR and SK have performed expert and guided gating. MR, SK, TK and GG have written the manuscript. All authors

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