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bjh short report

Flow cytometric minimal residual disease assessment in B-cell precursor acute lymphoblastic leukaemia patients treated with CD19-targeted therapies — a EuroFlow study

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Summary

The standardized EuroFlow protocol, including CD19 as primary B-cell marker, enables highly sensitive and reliable minimal residual disease (MRD) assessment in B-cell precursor acute lymphoblastic leukaemia (BCP-ALL) patients treated with chemotherapy. We developed and validated an alternative gating strategy allowing reliable MRD analysis in BCP-ALL patients treated with CD19-targeting therapies. Concordant data were obtained in 92% of targeted therapy patients who remained CD19-positive, whereas this was 81% in patients that became (partially) CD19-negative. Nevertheless, in both groups median MRD values showed excellent correlation with the original MRD data, indicating that, despite higher interlaboratory variation, the overall MRD analysis was correct.

Keywords: acute leukaemia, diagnostic haematology, flow cytometry, minimal residual disease.

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Introduction

Minimal residual disease (MRD) is used as an important prognostic biomarker in B-cell precursor acute lymphoblastic leukaemia (BCP-ALL) treatment protocols.[1] In Europe, MRD levels are generally assessed by real-time quantitative polymerase chain reaction (RQ-PCR) analysis of immunoglobulin and/or T-cell receptor gene rearrangements,[2] but in more recent protocols MRD is also being evaluated by flow cytometry.[3,4] A standardized protocol for flow cytometric MRD assessment in BCP-ALL was developed by the EuroFlow consortium.[5] This protocol includes the use of two eight-color antibody panels with CD19 as primary B-cell marker. In addition to antibody panels, also instrument settings and staining protocols were standardized. Together, these protocols make highly sensitive (down to 0·001%) and reliable MRD assessment in BCP-ALL possible, providing MRD data that are highly comparable to RQ-PCR methods.[5] It should however be noted that this comparison was made in the setting of classical chemotherapeutic treatment protocols.[5]

In recent years, novel targeted therapies have been developed, including therapies targeting CD19. Initially, Blinatumomab (CD19 × CD3) was approved in 2014 by the FDA as second-line treatment for Philadelphia chromosome-negative relapsed or refractory BCP-ALL.[6] Subsequently, chimaeric antigen receptor (CAR) T-cell therapies targeting CD19 were developed for the treatment of patients with relapsed or refractory BCP-ALL[7] and resulted in significantly improved outcome.[7,8] Besides the clinical improvements, these new therapeutic approaches may however interfere with the diagnostic CD19 antibodies in the eight-color BCP-ALL MRD panel and part of the patients may escape therapy through outgrowth of CD19-negative ALL cells.[9–11] Consequently, CD19-based identification of B-cells may not be sufficient in at least a subset of BCP-ALL patients treated with CD19-targeted therapies. Therefore, we aimed to develop an alternative gating strategy, without the use of CD19 as a B-cell-specific marker, in order to evaluate whether MRD could reliably be measured using the EuroFlow protocol in BCP-ALL patients treated with CD19-targeting therapies.

For this purpose, a multi-centre evaluation was performed in several phases. In the first phase, flow cytometry standard (FCS) files from 17 samples, obtained from 15 chemotherapy-treated BCP-ALL cases from different centres, were selected from our previous MRD study.[5] Samples obtained at day 15 and day 33 of therapy were selected, since relatively high MRD levels are present at both time points. CD19 fluorescence data were electronically removed from the

MRD FCS files. The resulting FCS files were distributed to the seven participating centres, together with the diagnostic FCS file, and were analyzed in Infinicyt Software (Cytognos, Salamanca, Spain). Using the original flow cytometry data (obtained using CD19 as primary gating marker and confirmed by RQ-PCR analysis) as reference, the newly-obtained MRD data were considered to be concordant if: (i) the MRD level was within a factor three of the reference value for MRD positive samples, or (ii) the MRD result was negative in both the original and the new (CD19-omitted) analysis. In the first phase, a concordance of 86% was obtained; false-positivity was not obtained, but 3% (3/119) were scored as false-negative and in 6% (7/119) of the analyzed files the laboratory indicated that reliable MRD analysis was not possible (Table SI). Next, 17 additional cases, obtained at day 33 and day 78 (i.e. with relatively low or undetectable MRD levels), were selected. Again, CD19 fluorescence data were electronically removed from the FCS files. MRD analysis showed an overall concordance of 76% compared to the original flow cytometry data. False-negative MRD data were obtained in 8% (10/119) of the samples, mainly day 33 samples (Table SII). Nine percent (11/119) of cases were scored false-positive, which concerned mainly day 78 samples with relatively high levels of normal BCP regeneration. In 5% (6/119) of the analyzed files, reliable MRD analysis was found not to be possible. Given the data generated in these first two phases (overall concordance 81%), it was concluded that the eight-color BCP-ALL MRD tube can be used for MRD assessment, also in cases in which CD19 cannot be used as B-cell-specific gating marker, but that analysis guidelines were needed.

Therefore, a common gating strategy was discussed and designed and subsequently distributed between the participating centers (two additional centers were included in this third phase for a total of 9 laboratories). This gating strategy was evaluated using a new dataset containing 16 follow-up samples of BCP-ALL patients with variable MRD levels (Table S3). CD19 fluorescence data were electronically removed from the FCS files. In the first analysis, the immunophenotype of the BCP-ALL cells at diagnosis was not provided to the participating centres. An overall concordance of 73% was achieved; 8% (11/144) were scored as false-positive, 10% (15/144) were scored false-negative and in 3% (4/144) of cases reliable MRD analysis was considered not to be possible (Table SIII). Subsequently, the diagnostic immunophenotyping data were provided to the participating centres and the same 16 follow-up samples were reanalysed using the same common strategy. Knowledge of the diagnostic immunophenotype improved the results to an overall concordance of 81%; 6% (8/144) of the samples were scored false-positive and 10% (14/144) of the samples were scored false-negative (Table SIV). Based on the experiences obtained with the common gating strategy, this strategy was critically discussed and slightly modified accordingly (Fig 1). In addition, it was concluded that the use of reference images, based on the immunophenotype of various normal BCP subsets (pre-B-I, pre-B-II, immature, transitional) and mature B-cells, was highly recommended for the MRD analysis. The analysis template including such reference images, already used in part of the analysis, was made available to all participants and can also be downloaded from the EuroFlow website (www.euroflow.org).

The modified strategy was first evaluated using the same 16 patient samples of the previous phase. Participating laboratories were requested to analyze these data without taking the previous data into account ("blinded") or by another staff member. Using the modified strategy, a concordance of 92% was achieved; 2% (3/144) were scored false-positive and 3% (4/144) of the cases were scored false-negative (Table SV). These data suggest that the modified gating strategy and the use of reference images improved the MRD analysis, although it cannot be excluded that some improvement was due to the previously obtained experience with the data set used.

Finally, the modified gating strategy was validated in a new "real life" cohort, composed of 36 MRD samples from seven different centres. Eighteen patients were treated with chemotherapy and 18 patients were treated with CD19-targeted therapies. Of the latter patients, ten showed a (partially) CD19-negative immunophenotype (Table SVI). Anonymized FCS data files (including CD19) were distributed to the different participating centres and MRD levels were determined. Overall, a concordance of 89% was achieved. For the chemotherapy-treated patients, the concordance was 92%; 1% (2/162) scored false-positive and 6% (9/162) scored false-negative (Table SVI). These data are comparable to the previously reported concordance applying the EuroFlow BCP-ALL approach (93%).[5] For the targeted therapy cases the concordance was comparable (86%; $P = 0{\cdot}11$ by chi-square test); 1% (3/162) scored false-positive and 6% (10/162) scored false-negative (Table SVI). The concordance was 92% in patients who remained CD19-positive, whereas this was 81% in patients that had become (partially) CD19-negative ($P = 0{\cdot}03$ by chi-square test). The frequency of false-negative data was not significantly different between both groups ($P = 0{\cdot}11$ by chi-square test). In both treatment groups (chemo/targeted), original and re-analyzed MRD levels showed very good correlations (Fig 2) indicating that, despite higher interlaboratory variation (particularly in two cases), the overall MRD analysis was correct. Therefore, analysis of MRD in BCP-ALL patients using the eight-color EuroFlow tubes can reliably be done, both in patients treated with chemotherapy and in patients treated with CD19-targeted therapies.

Although the modified gating strategy is suitable for MRD analysis in the vast majority of the BCP-ALL patients, it likely remains more difficult to assess MRD levels in CD10-negative B-ALL (i.e. pro-B-ALL and some pre-B-ALL) patients treated with targeted therapies, especially if these are also CD34-negative.[12] Such cases were only limitedly included in our 'real life' cohort (Table SVII). Reference images of normal B-cell precursors as well as knowledge of

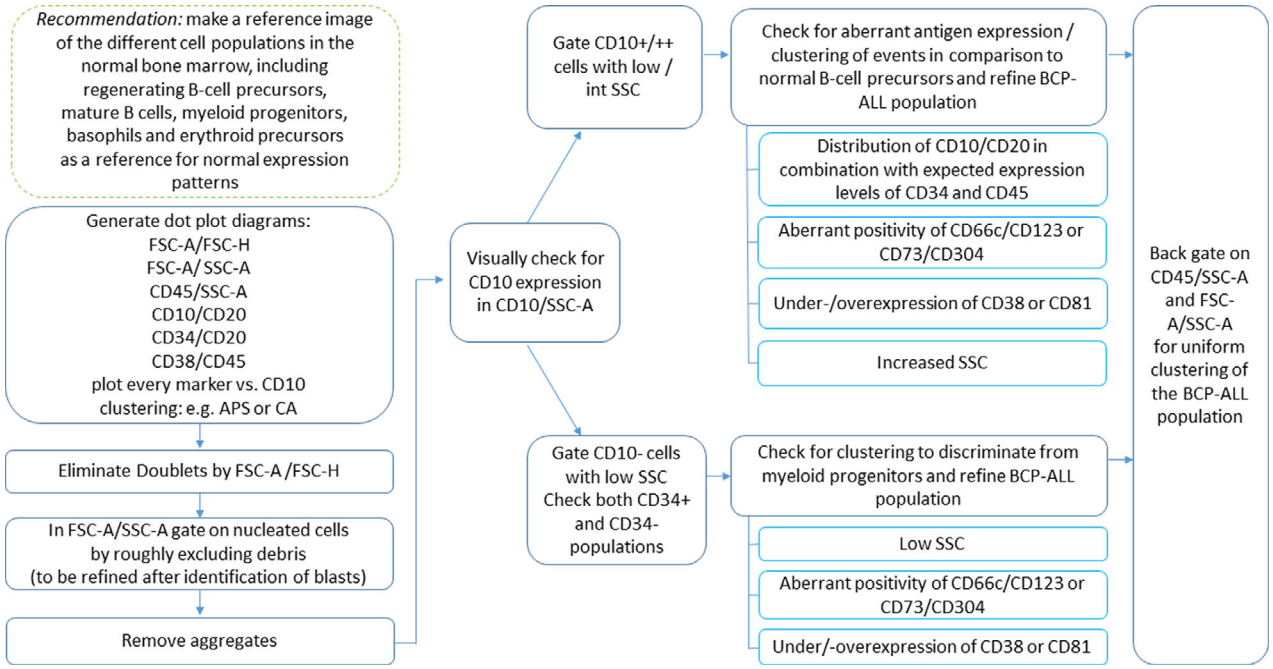

Fig 1. Gating strategy for MRD assessment of B-cell precursor acute lymphoblastic leukaemia (BCP-ALL) patients treated with CD19-targeted therapies. After initial gating of nucleated, single cells and exclusion of debris and aggregates, an initial evaluation is based on the possible presence of $CD10^+$ lymphocytes. If present, these cells are further evaluated for abnormal expression patterns (with help of reference images) to define whether they are ALL cells or not. If $CD10^+$ lymphocytes were not present, further analysis is first focused on CD34. If $CD34^+$ cells are present these can further be evaluated for abnormal expression patterns. Subsequently, also the $CD34^-$ lymphocytes are being evaluated for aberrant expression patterns. In all cases, possible ALL cells are ultimately back-gated on the forward scatter (FSC)-side scatter (SSC) and CD45-SSC plot to check that the cells form a uniform cluster. [Colour figure can be viewed at wileyonlinelibrary.com]

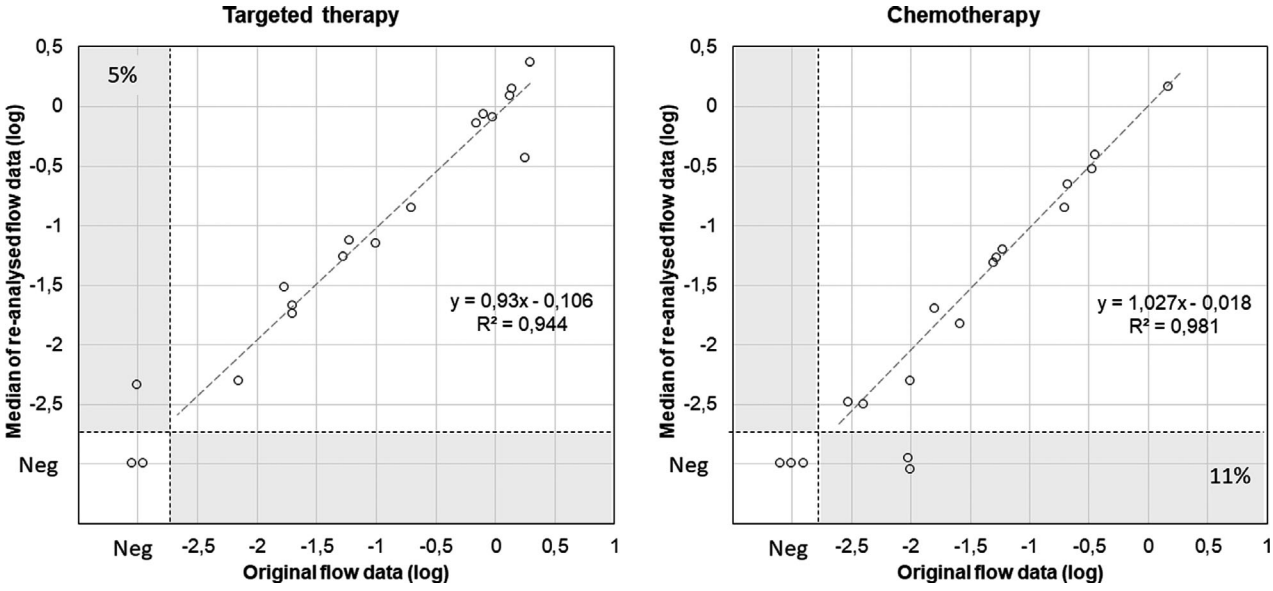

Fig 2. Comparison of original MRD levels and MRD levels re-analysed in phase 6. All MRD data were log10-transformed before plotting and calculating correlation coefficients. Each symbol reflects the original MRD level *versus* the median of the re-analysed MRD data by the participating centres ($n = 9$). In the targeted therapy group (left panel), one sample (5%) scored negative in the original analysis but was scored low level MRD positive in the re-analysis. Molecular MRD data showed a level of 4×10^{-4} (Table SVI). In the chemotherapy group (right panel), two samples (11%) were scored negative in the re-analysis but were originally scored positive (both at 0·01%). Molecular data were 2×10^{-4} for both samples (Table SVI). The correlation of the samples in which both MRD data were positive is shown in the plots (dotted line with equation and correlation coefficient).

the original immunophenotype of the patient at diagnosis facilitates the MRD analysis (in phase 6, concordance was 91% for cases with this information available *versus* 84% for cases without). It should also be noted that in our study the laboratories only received the FCS files, without any other information about the patient (except for original immunophenotype in part of the patients). In practice, additional information about the patient will generally be available (e.g. treatment, timepoint in protocol, previous MRD data) or can become available (e.g. by extra stainings including additional markers). The availability of such information will also facilitate the MRD analysis.

Further technical improvements, e.g. expanding the current eight-color panel with additional B-cell markers such as CD22 and CD24,[13] and software improvements, e.g. database-supported analysis using automated gating and identification of normal and abnormal B-cells,[9–11] are currently being evaluated within the EuroFlow consortium. Until these improvements become available, the here presented gating algorithm can be used to analyze MRD data from patients treated with CD19-targeted therapies by using the EuroFlow BCP-ALL MRD protocol. However, careful training and availability of pretreatment immunophenotypes are required and one should be particularly cautious when analysing CD10- and/or CD34-negative cases.

Conflict of interest

JJMvD, AO, JFM, TS and VHJvdV each report being one of the inventors on the EuroFlow-owned patent PCT/NL2010/050332 (Methods, reagents and kits for flow cytometric immunophenotyping of normal, reactive and malignant leukocytes). The Infinicyt software is based on intellectual property of some EuroFlow laboratories (University of Salamanca in Spain and Federal University of Rio de Janeiro in Brazil) and the scientific input of other EuroFlow members. All above-mentioned intellectual property and related patents are licensed to Cytognos (Salamanca, Spain) and BD Biosciences (San José, CA, USA), which companies pay royalties to the EuroFlow Consortium. These royalties are exclusively used for continuation of the EuroFlow collaboration and sustainability of the EuroFlow consortium. VHJvdV reports a Laboratory Services Agreement with BD Biosciences; all related fees are for the Erasmus MC. JJMvD and JAOMCV report an Educational Services Agreement from BD Biosciences and a Scientific Advisor Agreement with Cytognos; all related fees and honoraria are for the involved university departments at Leiden University Medical Centre and University of Salamanca. MB received personal fees from Incyte (advisory board) and Roche Pharma AG, financial support for reference diagnostics from Affimed, Amgen, BMS and Regeneron, grants and personal fees from Amgen (advisory board, speakers bureau, travel support), and personal fees from Janssen (speakers bureau), all outside the submitted work. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

Conception or design of the work: GG, VHJvdV. Data collection: MWCV, CB, AL, SN, LS, JFM, MH, ESdC, MN, EM, SBD, SK, EO, RJ, AXdJ, TS, JP, MB, GG, VHJvdV. Data analysis and interpretation: MWCV, CB, AL, SN, LS, JFM, MH, ESdC, MN, EM, SBD, SK, EO, RJ, AXdJ, TS, JP, JJMvD, AO, MB, GG, VHJvdV. Drafting the article: MWCV, CB, GG, VHJvdV. Critical revision of the article: all authors. Final approval of the version to be published: all authors.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table SI. Results obtained after phase 1 of MRD analysis in seven participating centres.[a]

Table SII. Results obtained after phase 2 of MRD analysis in seven participating centres.[a]

Table SIII. Results obtained after phase 3 of MRD analysis in nine participating centres.[a]

Table SIV. Results obtained after phase 4 of MRD analysis in nine participating centres.[a]

Table SV. Results obtained after phase 5 of MRD analysis in nine participating centres.[a]

Table SVI. Results obtained after phase 6 of MRD analysis in nine participating centres.[a]

Table SVII. Immunophenotype of BCP-ALL cases included in the various phases of the study.

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