Automate more.
Get more done.

Reduce hands-on time with a true walkaway flow cytometry solution that frees up your team to:

- focus on analysis
- be more efficient
- **keep up with testing demands**
- focus on more critical lab tasks
- make the most of the workday

Drive efficiency from sample to answer with the power of the fully automated BD FACSDuet™ Premium Sample Preparation System, offering cocktailing, washing, centrifugation and sample transfer to a physically integrated flow cytometer.

Get more done. Discover how at **bdbiosciences.com/duet**

BD, the BD Logo, BD FACSDuet and BD FACSLyric are trademarks of Becton, Dickinson and Company or its affiliates. © 2023 BD. All rights reserved. BD-88682 (v.1) 0523

Check for updates

Received: 23 December 2022 | Revised: 28 July 2023 | Accepted: 6 September 2023
DOI: 10.1002/cyto.b.22143

ORIGINAL ARTICLE

CLINICAL CYTOMETRY WILEY

Minimal residual disease assessment in B-cell precursor acute lymphoblastic leukemia by semi-automated identification of normal hematopoietic cells: A EuroFlow study

Martijn W. C. Verbeek[1] | Beatriz Soriano Rodríguez[2,3,4,5] | Lukasz Sedek[6,7] | Anna Laqua[8] | Chiara Buracchi[9] | Malicorne Buysse[10,11] | Michaela Reiterová[12] | Elen Oliveira[13] | Daniela Morf[14] | Sjoerd R. Oude Alink[1] | Susana Barrena[2,3,4,5] | Saskia Kohlscheen[8] | Stefan Nierkens[15] | Mattias Hofmans[10,11] | Paula Fernandez[14] | Elaine Sobral de Costa[13] | Ester Mejstrikova[12] | Tomasz Szczepanski[7] | Lukasz Slota[7] | Monika Brüggemann[8] | Giuseppe Gaipa[9] | Georgiana Grigore[16] | Jacques J. M. van Dongen[2,3,4,5,17] | Alberto Orfao[2,3,4,5] | Vincent H. J. van der Velden[1]

[1]Laboratory for Medical Immunology, Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands
[2]Translational and Clinical Research program, Cancer Research Centre (IBMCC, CSIC-USAL), Cytometry Service, NUCLEUS, Salamanca, Spain
[3]Department of Medicine, University of Salamanca (USAL), Salamanca, Spain
[4]Institute of Biomedical Research of Salamanca (IBSAL), Salamanca, Spain
[5]Biomedical Research Networking Centre Consortium of Oncology (CIBERONC), Instituto de Salud Carlos III, Madrid, Spain
[6]Department of Microbiology and Immunology, Medical University of Silesia in Katowice, Zabrze, Poland
[7]Department of Pediatric Hematology and Oncology, Zabrze, Medical University of Silesia in Katowice, Katowice, Poland
[8]Department of Hematology, University of Schleswig-Holstein, Kiel, Germany
[9]Centro Tettamanti, Fondazione IRCCS San Gerardo dei Tintori, Monza, Italy
[10]Department of Diagnostic Sciences, Ghent University, Ghent, Belgium
[11]Department of Laboratory Medicine, Ghent University Hospital, Ghent, Belgium
[12]CLIP-Department of Pediatric Hematology and Oncology, Second Faculty of Medicine, Charles University and University Hospital Motol, Prague, Czech Republic
[13]Pediatrics Institute IPPMG, Faculty of Medicine, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil
[14]Institute for Laboratory Medicine, Aarau, Switzerland
[15]Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands
[16]Cytognos SL, Salamanca, Spain
[17]Department of Immunology, Leiden University Medical Center (LUMC), The Netherlands

Correspondence
Vincent H.J. van der Velden, Department of Immunology, Erasmus MC, Doctor Molewaterplein 40, 3015 GD Rotterdam, The Netherlands.
Email: v.h.j.vandervelden@erasmusmc.nl

Abstract

Presence of minimal residual disease (MRD), detected by flow cytometry, is an important prognostic biomarker in the management of B-cell precursor acute lymphoblastic leukemia (BCP-ALL). However, data-analysis remains mainly expert-dependent. In this study, we designed and validated an Automated Gating & Identification (AGI) tool

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2023 The Authors. *Cytometry Part B: Clinical Cytometry* published by Wiley Periodicals LLC on behalf of International Clinical Cytometry Society.

Funding information
Fondo Europeo de Desarrollo Regional (FEDER); European Commission, Grant/Award Number: LSHB-CT-2006-018708; CIBERONC, Grant/Award Number: CB16/12/00400; Instituto de Salud Carlos III (ISCIII), Grant/Award Number: PI19/01183; Ministry of Health of the Czech Republic, Grant/Award Number: NU20J-07-00028; National Institute for Cancer Research, Grant/Award Number: LX22NPO5102; European Union - Next Generation EU; Deutsche José Carreras Leukämie-Stiftung, Grant/Award Numbers: DJCLS R 15/11, DJCLS 06R/2019; Medical Univeristy of Silesia, Grant/Award Number: PCN-1-075/K/1/K; Fondazione M. Tettamanti M. De Marchi ONLUS

for MRD analysis in BCP-ALL patients using the two tubes of the EuroFlow 8-color MRD panel. The accuracy, repeatability, and reproducibility of the AGI tool was validated in a multicenter study using bone marrow follow-up samples from 174 BCP-ALL patients, stained with the EuroFlow BCP-ALL MRD panel. In these patients, MRD was assessed both by manual analysis and by AGI tool supported analysis. Comparison of MRD levels obtained between both approaches showed a concordance rate of 83%, with comparable concordances between MRD tubes (tube 1, 2 or both), treatment received (chemotherapy versus targeted therapy) and flow cytometers (FACSCanto versus FACSLyric). After review of discordant cases by additional experts, the concordance increased to 97%. Furthermore, the AGI tool showed excellent intra-expert concordance (100%) and good inter-expert concordance (90%). In addition to MRD levels, also percentages of normal cell populations showed excellent concordance between manual and AGI tool analysis. We conclude that the AGI tool may facilitate MRD analysis using the EuroFlow BCP-ALL MRD protocol and will contribute to a more standardized and objective MRD assessment. However, appropriate training is required for the correct analysis of MRD data.

KEYWORDS
automation, B-cell differentiation, BCP-ALL, database, MRD

1 | INTRODUCTION

Minimal residual disease (MRD) is used as an important prognostic biomarker in the management of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) (Gaipa et al., 2013). In current protocols, MRD is either assessed by molecular analysis (e.g., B- and T-cell receptor gene rearrangements) or by flow cytometry (van Dongen et al., 2015). A standardized operating procedure (SOP) for 8-color next generation flow cytometric MRD assessment in BCP-ALL was developed by the EuroFlow consortium (Theunissen et al., 2017). This SOP includes protocols for standardized instrument settings, standardized sample processing, data acquisition for >10^6 cells, and two standardized 8-color antibody tubes (Theunissen et al., 2017). This SOP allows sensitive MRD detection in virtually all BCP-ALL patients. However, data-analysis remains mainly expert dependent and requires comprehensive and extensive training to obtain concordant results (Maurer-Granofszky et al., 2021). In addition, the EuroFlow protocols were designed and optimized using patients treated with classical chemotherapy (Theunissen et al., 2017), while in the meantime novel targeted therapies (mainly therapeutic antibodies and chimeric antigen receptor (CAR) T-cells) have been developed for the treatment of BCP-ALL patients (Lu et al., 2021; Singh et al., 2008). These novel therapies result in a significantly improved outcome compared to chemotherapy treated patients (Harris et al., 2021), but may also interfere with the diagnostic antibodies in the BCP-ALL MRD panel (Mikhailova et al., 2021). In addition, use of targeted therapies may result in the outgrowth of CD19-negative ALL cells (Bueno et al., 2022; Libert et al., 2020; Mikhailova et al., 2021), requiring alternative gating strategies for appropriate MRD analysis (Verbeek et al., 2022). Altogether, gating and identification of MRD cells becomes even more complex and therefore software-supported data analysis may facilitate MRD assessment.

In recent years, various software tools, including machine learning-based algorithms, have been developed to (semi)automatically analyze flow cytometry data (Lhermitte et al., 2021; Shopsowitz et al., 2022). These algorithms use different approaches to identify different hematopoietic cell subsets in flow cytometry data. Algorithms have been designed using a combination of multiple Gaussian Mixture Models (Reiter et al., 2019), neural network approaches (Wodlinger et al., 2022), t-Distributed Stochastic Neighbor Embedding-based methods (tSNE) (DiGiuseppe et al., 2015), radar plots (Shopsowitz et al., 2022), hierarchical clustering (Fiser et al., 2012) and database-based algorithms (Flores-Montero et al., 2017; Lhermitte et al., 2021). With the database-based approach, different cell clusters are first recognized and identified within Flow Cytometry Standard (FCS) files, and subsequently compared to the various normal cell populations stored in a database of normal/regenerating bone marrow cells located in a multidimensional space defined by their light scatter properties and the fluorescent intensities of multiple markers in the individual cells in each cell population in the database (Pedreira et al., 2013; Pedreira et al., 2019). Such semi-automated identification of hematopoietic cell subsets will result in a more objective and less expert-dependent analysis of flow cytometry data (Flores-Montero et al., 2017; Lhermitte et al., 2021; Reiter et al., 2019). Within the EuroFlow consortium, Automated Gating & Identification (AGI) tools have been developed, among other applications, for the classification of acute leukemia's (Lhermitte et al., 2021), plasma cell malignancies (Flores-Montero et al., 2017),

15524957, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cyto.b.22143 by Cochrane Netherlands, Wiley Online Library on [29/09/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

chronic lymphoproliferative disorders (Flores-Montero et al., 2019), primary immunodeficiencies, and multiple myeloma (MM) MRD in both bone marrow and blood (Linskens et al., 2020). However, there is no dedicated AGI tool developed for analysis of the 8-color BCP-ALL MRD panel yet, while such tool may facilitate data analysis and may also contribute to a more standardized analysis of flow cytometry data. In this study we designed an AGI tool for identification of normal hematopoietic cell subsets in bone marrow (BM) using the 8-color BCP-ALL MRD panel. A reference database of normal BM samples stained with the BCP-ALL MRD panel was built and the AGI tool was validated in a multi-center validation cohort consisting of over 170 data files, obtained from patients treated with chemotherapy or targeted therapies and acquired on FACSCanto or FACSLyric flow cytometers.

2 | METHODS

2.1 | Normal FCS-files for the BCP-ALL MRD database

For the construction of the BCP-ALL MRD database, FCS-files from 68 normal BM samples, processed and stained according to the EuroFlow BCP-ALL MRD protocol (Theunissen et al., 2017) in 10 participating centers, were used. FCS-files were included in the database if the BM aspirate was obtained from a healthy volunteer or stem cell donor ("healthy", $n = 29$), patients without immunological or hematological diseases ("normal patient", $n = 4$), leukemia/lymphoma patients in long-term complete remission ("L/L patient in CR", $n = 13$) or patients with immunological disorders (e.g., immune thrombocytopenia) without a hematological malignancy ("other patient", $n = 21$); details from one case were unknown.

2.2 | Database building

A EuroFlow analysis profile was defined using Infinicyt software (version 2.0.5; Cytognos, Salamanca, Spain). In this analysis profile, 15 cell populations were included, defined according to their specific immunophenotypic profile obtained using the BCP-ALL MRD EuroFlow panel (Table S1). These included: pro-B-cells, pre-B1-cells, pre-B2-cells, immature CD10+ B cells, mature B-cells, plasma cells, additional CD34+ precursors other than B-cell precursors, eosinophils, neutrophils, monocytes, T and NK cells, nucleated red cells, mesenchymal stem cells, endothelial cells and unspecified nucleated cells (including basophils and dendritic cells). In addition, cell debris and doublets (mainly identified by their forward (FSC) and sideward (SSC) light scatter characteristics) were defined. Each FCS-file was analyzed using this profile and the technical quality of each file was evaluated with the Infinicyt software to guarantee optimal quality of the files (see Results). Finally, for each case included in the database, the age of the donor was registered and three different age categories were defined: <5 years, 5–15 years and >15 years. For one of the donors age was not available, the data of this individual therefore were not used for age-dependent analysis.

2.3 | Automated gating and identification tool

For the normal BM samples, the included files were stored in the database module of the Infinicyt software; separate databases were constructed for tube 1, tube 2, and the merged tubes. Age-dependent thresholds for alerts were calculated using 5–95 percentile statistics of the percentage of populations and were introduced into the Infinicyt software. A profile containing numeric alerts and an automated report was also designed. The AGI-supported analysis consists of different steps. First, the AGI tool is looking for clusters of cells. The applied clustering algorithms used had the following characteristics: ≥10 events were required per cluster (i.e., *K* parameter) at a maximum distance/dispersion within the cluster of 0.9 (i.e., S parameter, arbitrary units), as previously described in detail elsewhere (Rodriguez et al., 2017). Next, the AGI tool compares the multidimensional immunophenotypic patterns of the identified clusters with the immunophenotypic information stored in pre-defined canonical analysis (CA) for all possible comparisons of each pair of the 15 normal cell populations identifiable in normal bone marrow with the BCP-ALL MRD panel. Depending on the degree of concordance, the AGI tool classified all clusters of events by assigning each cluster that was identical to a cell population in the database directly as phenotypically normal cells from that cell population in the data base they are identical to. For all other clusters that were not directly assigned to one of the pre-defined cell populations in the normal (control) bone marrow database, because despite being similar to a given cell population in the data base they were not identical to it, they were on purpose, assigned as checks for final review by an expert with an indication about the closest cell populations in the database (Flores-Montero et al., 2017; Lhermitte et al., 2021; Linskens et al., 2020). In the final step, the checks defined by the AGI tool classified per cell population in the database had to be reviewed by the expert-user for final assignment to either a normal population or to the abnormal (ALL) population. In this step, the expert-user evaluates the immunophenotype of the check population and based on the characteristics of the cells (expression of B-cell markers, expression of maturation markers, expression of aberrant markers) and the experience/knowledge of the user the cells are either defined as normal or abnormal, independently of the automated classification proposed by the AGI tool. It is expected that the ALL cells will be included in a cluster that is different from normal and typically similar to one of the distinct populations of B-cell precursors in the database; consequently they will be flagged as check population(s).

2.4 | Samples used to validate the AGI software tool

To evaluate the accuracy of the AGI tool for BCP-ALL MRD analysis, a validation cohort was created based on available data files from our

15524957, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cyto.b.22143 by Cochrane Netherlands, Wiley Online Library on [29/09/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

previous studies (Theunissen et al., 2017; Verbeek et al., 2022), extended with more recently collected cases acquired on FACS Lyric flow cytometers (BD Biosciences, San Jose, CA). This validation cohort was composed of FCS-files obtained from BCP-ALL patients during follow-up and acquired using the EuroFlow BCP-ALL MRD SOPs at the ten participating centers. Overall, 174 data files were included: 125 samples were obtained from chemotherapy treated patients, 42 from targeted therapy treated patients, from 7 patients therapy was unknown; 127 samples were measured on FACSCanto II and 47 on FACSLyric flow cytometers. All files were analyzed without knowledge of patient therapy history, and flow cytometer used. In the optimal situation, a BCP-ALL MRD sample is stained with both tube 1 and tube 2. In the real-life situation the number of cells is most of the time insufficient to stain for both tubes since molecular MRD analysis generally has priority. When this is the case, the tube with the most informative antibody combination is used. In most cases CD66c and CD123 (tube 1) are more informative than CD73 and CD304 (tube 2), data obtained with tube 1 surpassed data obtained with tube 2 (112 versus 47, respectively).

2.5 | Validation of the AGI software tool

To validate the AGI software tool, anonymized FCS-files were run through the AGI tool, which was incorporated in the Infinicyt software and the resulting CYT-files were subsequently distributed among the ten participating centers. At these centers, all 'checks' were reviewed and MRD levels were determined. The MRD levels achieved by using the AGI tool plus expert review were compared with the original manually analyzed MRD levels. Discordant cases were blindly re-analyzed manually and by the AGI tool by four experts in eight different centers, without knowledge of previous results.

In addition, a selected number of cases (n = 25) was used for a full manual analysis of all identifiable hematopoietic subsets, and results (percent values) were compared with those obtained with the AGI tool. Furthermore, the inter-expert reproducibility was evaluated by analysis of ten AGI tool processed files by four experts from three different centers. From this analysis, mean and percent coefficient of variation (%CV) values were calculated. Finally, the repeatability of the AGI tool was validated by repeated analysis of ten AGI tool processed FCS-files five times by the same expert. Mean and %CV were calculated from all ten samples. For both the manual analysis and for the AGI-tool-supported analysis a similar sensitivity was reached, that is, 10^{-5} if at least 4 million events were acquired.

2.6 | MRD report

A standardized report, including the cellular composition (with alerts for percentages outside the age-dependent ranges) and immunophenotype of the abnormal population (if present) was designed and included in the profile. After assigning all checks, this report is automatically generated.

2.7 | Statistical analyses

All figures were generated in GraphPad Prism (version 9.0.0) and statistics were calculated with the incorporated tools of this software. Differences between percentages of doublets and debris as defined by both methods were calculated by paired t-tests. Correlations of MRD levels and percent cell subsets between manual analysis and AGI tool were calculated using the Pearson's correlation of log10 transformed MRD levels or other cell numbers, respectively. Differences between age groups and percentages of checks in selected groups were evaluated by ANOVA with Tukey post-hoc tests. To evaluate possible systematic differences, MRD levels determined by manual analysis or the AGI tool were compared using Bland–Altman analysis.

3 | RESULTS

3.1 | Database construction

Databases of normal BM samples stained and acquired according to the EuroFlow BCP-ALL MRD SOP were built for tube 1, tube 2 and for the combined tubes. The FCS-files from the 68 samples included in the databases were manually analyzed using Infinicyt software and all cases were critically reviewed. Subsequently, 44/68 (65%) cases were excluded for the generation of the database since they did not fulfill our stringent quality criteria due to the following issues: (i) wrong scatter threshold and/or inappropriate scatter setting that placed the lymphocytes in different positions than expected ($n = 21$), (ii) compensation problems (often involving the APC-A750 channel) ($n = 11$), (iii) possible pipetting errors of individual liquid reagents that led to absence or diminished expression of expected markers on normal populations, particularly related to CD20, CD38 and CD81 ($n = 13$), (iv) unstable acquisition in time identified by visualizing parameter vs. time and observing the gaps ($n = 3$). Furthermore, some files were excluded because of the lack of or reduced numbers of specific cell populations ($n = 7$; see Table S2). Finally, a database was constructed which consisted of 24 normal BM samples (Table S3). Sample donors had a median age of 20 years (4 <5 years; 6 between 5 and 15 years; 14 >15 years and 1 unknown). Of the 24 normal BM files, 18 FCS-files were obtained from FACSCanto II, and 6 FCS-files were obtained from FACSLyric instruments.

3.2 | Review of the flow cytometric files in the database

After generating the database and the clustering algorithms (see Methods), the AGI tool was first tested against the same FCS-files used for the generation of the database. As expected, virtually all events in the data files (96.4% and 97.3% in tube 1 and tube 2 patient data files) were automatically classified into one cell population, debris or cell doublets, with very low percentages of checks for the tube

15524957, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cyto.b.22143 by Cochrane Netherlands, Wiley Online Library on [29/09/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

1 files (mean: 3.6%, SD: 2.0%) and the tube 2 files (mean: 2.7%, SD 1.5%). Interestingly, the AGI tool-analyzed files showed slightly higher levels of debris compared to the manual analysis for tube 1 files (9.2% vs. 6.7%, $p < 0.001$) and tube 2 (7.4% vs. 5.4%, $p < 0.001$). Similar results were found for the levels of doublets between files analyzed with the AGI tool versus manual analysis for tube 1 (3.8% vs. 2.9%, $p < 0.001$) and tube 2 (4.0% vs. 2.9%, $p < 0.001$). Most importantly, the percentages of normal hematopoietic cell subsets as identified by the AGI tool highly correlated with the percentages obtained by manual analysis (Figure S1). These data show that the AGI tool appropriately gates and identifies normal cell subsets in control bone marrow samples with few checks classified per cell population to be reviewed by the expert.

3.3 | Cellular composition of normal BM samples

The FCS-files used for the generation of the database allowed us to determine the cellular composition of normal BM in relation to the age of the subject. Subjects were divided in three groups: children <5 years (which show substantial kinetics in B-cell numbers in peripheral blood), children between 5 and 15 years (with lower B-cell numbers almost comparable to adults), and subjects over 15 years of age (Comans-Bitter et al., 1997). In control subjects over 15 years of age, the proportion of total B-cells was significantly decreased compared to controls <15 years of age (Figure 1). In more detail, pro-B, pre-B1-cells, pre-B2-cells, immature CD10+, and mature B-cells showed lower frequencies in subjects over 15 years of age compared to younger subjects ($p < 0.05$). In addition, the B-cell compartment of controls >15 years of age showed a bias toward a more matured B-cell compartment compared to younger controls (Figure 1). Besides the differences in the B-cell compartment, the levels of monocytes were lowest in subjects >15 years of age compared to younger controls (especially those <5 years of age). Levels of endothelial cells were the lowest in subjects >15 years of age compared to subjects aged 5–15 years (Figure S2). In contrast, the levels of neutrophils were higher in controls >15 years of age compared to the youngest group of controls. No significant changes were found for the other cell subsets between the different age groups (Figure S2). These data show that the cellular composition of the BM is age-dependent, with increasing frequencies of neutrophils and decreasing frequencies of monocytes, mature B-cells and precursor-B-cells over time. Consequently, age-related thresholds for alerts were incorporated in the AGI tool.

3.4 | Validation of the AGI tool

To validate the accuracy of the AGI tool, 174 BCP-ALL MRD FCS-files were initially manually analyzed at one of the participating centers. Subsequently, these FCS-files were processed by the BCP-ALL MRD AGI tool and checks were reviewed by a different expert. One hundred three samples were determined to be MRD positive; before review, these MRD cells were assigned to the checks in all cases. In 57% of the MRD-positive files, MRD cells were marked as checks with nearest counterpart being B-cells; in 27% of cases MRD cells were assigned as checks nearest to debris; in the remaining 16%, MRD cells were assigned to other nearest populations.

The MRD levels obtained by manual analysis were correlated with MRD levels found by using the AGI tool. Eighty-nine files were marked as MRD positive by both approaches and 55 files were MRD negative by both approaches, resulting in an overall qualitative concordance of 83%. Fourteen files were identified as MRD negative by manual analysis but MRD positive by the AGI tool analysis. Conversely, 16 files were MRD positive by manual analysis but they were scored negative by AGI tool MRD assessment. For the samples determined MRD positive by both methods, log10-transformed MRD levels showed a good correlation ($R^2 = 0.8919$, $p < 0.0001$; Figure 2a). A Bland–Altman comparison showed a negligible bias of 0.001197 (SD = 1.157) between the manual analysis and the AGI tool and did not show a bias in relation to the level of MRD (Figure S3a). If a cutoff of 0.01% was used (as in some other studies (Reiter et al., 2019; Shopsowitz et al., 2022)) the concordance between both methods increased to 87%.

3.5 | Impact of type of therapy on the accuracy of AGI analyses

To gain insight into the impact of treatment on the accuracy of the BCP-ALL MRD AGI tool, correlations were calculated based on treatment received by individual patients. One hundred twenty-five patients had been treated with chemotherapy prior to the BM sampling and 42 patients were treated with targeted therapy (blinatumomab, rituximab, CD19 targeted CAR-T cells and/or inotuzumab). Sixty-five out of the 125 files of the chemotherapy group (52%) were found to be MRD-positive by both manual analysis and the AGI tool with a good correlation between the levels of residual BCP-ALL identified ($R^2 = 0.8178$, $p < 0.0001$). Forty out of 125 files (32%) were concordantly MRD-negative by both methods, resulting in an 84% qualitative concordance between both methods (Figure 3a). In comparison, 21 out of 42 targeted therapy treated patients (50%) were found to be MRD-positive by both methods, with a strong correlation between the MRD levels detected ($R^2 = 0.9158$ $p < 0.0001$), 7 of these 21 MRD-positive patients showed CD19-negative ALL cells. Finally, 13 out of 42 files were found to be MRD negative by both manual and AGI tool analysis, resulting in an 81% overall qualitative concordance between the two methods (Figure 3b). These data show that the AGI tool is suitable for both chemotherapy as well as targeted therapy treated patients.

3.6 | Impact of the BCP-ALL MRD antibody combination and flow cytometer on the accuracy of the AGI-tool

In addition to the distinction made based on treatment history, the performance of the AGI-tool was evaluated separately for each of the two antibody combinations (tubes) of the BCP-ALL MRD panel used. In addition, it was evaluated if the AGI-tool is suitable for files

15524957, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cyto.b.22143 by Cochrane Netherlands, Wiley Online Library on [29/09/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

FIGURE 1 Distribution of bone marrow hematopoietic cell subsets between control subjects of different age categories. Percentages of different cell subsets relative to the total amount of nucleated cells are shown for subjects <5 years ($n = 4$), between 5 and 15 ($n = 6$), and >15 years ($n = 13$). P-values calculated by ANOVA with Tukey post-hoc are shown *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

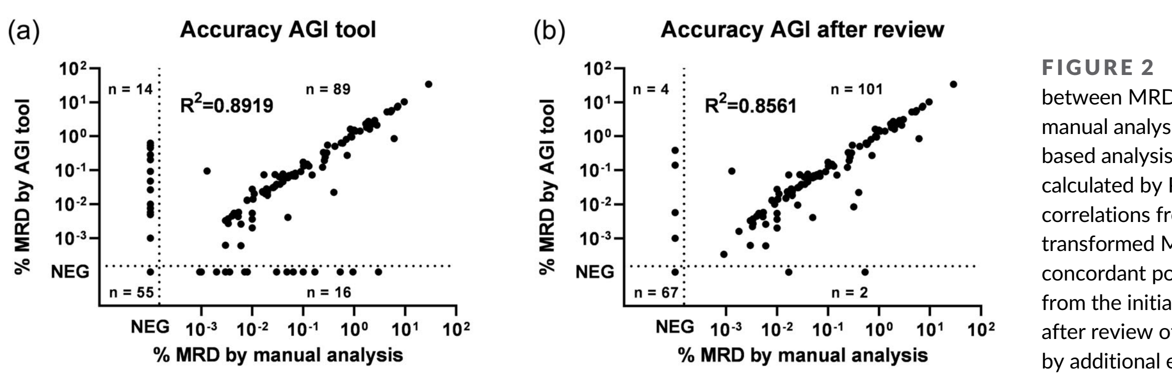
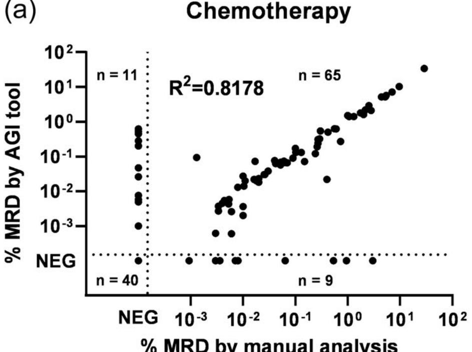


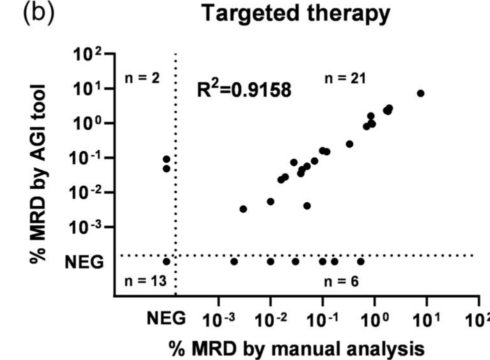
FIGURE 2 Correlation between MRD levels obtained by manual analysis vs. the AGI tool based analysis. The correlation is calculated by Pearson's correlations from log10 transformed MRD levels of the concordant positive files. (a) Data from the initial analysis. (b) Data after review of discordant cases by additional experts.

obtained from FACSCanto and the more recently introduced FACSLyric instruments, the rate of concordance and the degree of correlation between MRD results, were calculated for the files in the validation cohort dependent on the antibody combination and flow cytometer used (see Supplemental information S1 and Figure S4). Our data (Supplemental information S1 and Figure S4) indicate that the AGI tool performed comparably for FCS-files from tube 1 and/or 2 and show that the AGI tool performs equally well with data obtained from both instruments, supporting the correct inter-laboratory standardization of instrument settings according to EuroFlow protocols.

15524957, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cyto.b.22143 by Cochrane Netherlands, Wiley Online Library on [29/09/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

FIGURE 3 Correlation between MRD levels obtained by manual analysis vs. the AGI tool based analysis. Correlations are calculated by Pearson's correlations from log10 transformed MRD levels of the concordant positive files. Comparisons are shown for (a) chemotherapy and (b) targeted therapy treated patients.





3.7 | Evaluation of discordant cases

In the first round of analysis, 30 FCS-files (17%) showed discordant qualitative results between manual and AGI tool analysis. These discordant files were blindly reanalyzed by four other experts, both manually as well as by the AGI tool, resulting in a total of five independent measurements for both methods. Consensus between the five experts (i.e., same MRD indications (pos/neg) in 4 out of 5 results) was obtained for 12 cases by AGI tool (40%) and for 21 cases by manual analysis (70%). Taken the consensus data into account, an overall qualitative concordance between manual analysis and AGI tool was reached in 168 cases (97%) (Figure 2b). Bland–Altman analysis between two methods did show a minor systemic bias (0.02826, SD = 0.5727) (Figure S3b). Overall these data indicate that MRD assessment is comparable between manual analysis and the AGI tool and that appropriate training is required for correct analysis of MRD data, either by manual analysis or by AGI supported analysis.

3.8 | Percent checks in the validation cohort

For further evaluation of the AGI tool, the percentages of checks after its application were reviewed. Percentages were obtained from files from normal BM samples used to build the database, files from normal BM samples which were rejected for the database, patient files from the validation cohort which were MRD-negative, and files from patients that were strongly positive (>1% MRD). As expected, the normal BM files used to build the database had the highest percentages of directly classified events with the lowest percentages of checks (Figure S5). These percentages were comparable with the percentages of checks in MRD-negative patient samples (3.6% vs. 6.4%, $p > 0.05$). Files from rejected database files showed slightly higher percentages of checks (8.8%) compared to the included database files and the MRD-negative groups. FCS-files from patients with high MRD levels showed significantly higher percentages of checks (10%) compared to MRD-negative patient files and included, and excluded database files. Rejected database files and MRD-negative patient files showed several outliers with high percentages of checks (>10%). Detailed analysis of these FCS-files showed poor sample quality or the presence of abnormal cell subsets. Overall, these data indicate that the great majority (>90%) of events in a BM data file are automatically classified as normal cells by the AGI tool meaning that the percentage of checks for expert-review is generally low (<10%), except for the samples with high MRD levels or poor sample quality.

3.9 | Repeatability and reproducibility of the AGI tool-based analysis

To validate the intra-expert variation of the BCP-ALL MRD AGI tool, 10 samples were analyzed five times by the same expert (i.e. AGI tool was run, and cells in checks were assigned to the right population). Mean MRD values of the five repeated analyses were correlated with the original manual analysis of these 10 patient files (Figure 4a), and a nearly perfect correlation was observed between the two analysis strategies ($R^2 = 0.9999$, $p < 0.001$). All samples showed %CV values between 0.0% and 7.0% (median: 2.5%), indicating excellent repeatability of the AGI tool when the AGI tool results are reviewed by the same expert. In addition to the evaluation of the intra-expert variation of the BCP-ALL MRD AGI tool, the inter-expert variation was evaluated. Therefore, 10 patient FCS-files were reviewed after AGI tool analysis by four different experts from multiple centers. Overall, these samples showed a quantitative concordance of 90% (Figure 4b). In total 36 out of 40 (90%) analyzes gave highly comparable results, whereas four analyses (from three samples) had discordant results. For the seven samples with full concordant qualitative results, the % CV values were below 27% (range 0%–26.6%, median: 3.1%).

3.10 | Correlations between manual and AGI tool-based analysis of normal cell populations in BCP-ALL bone marrow samples

To evaluate the accuracy of the AGI tool for the identification of normal hematopoietic subsets in BM obtained from BCP-ALL patients during follow-up, FCS-files from 25 patients were randomly selected. Hematopoietic subsets were manually identified in these 25 FCS-files and files were analyzed by the AGI tool followed by assignments of the check populations. Of the B-cell subsets, pro-B-cells, pre-B1-cells, pre-B2-cells, mature B-cells and plasma cells showed highly concordant percentages of cell subsets between log10 transformed results from manual analysis and AGI tool analysis (Figure 5). Also, most non-

15524957, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cyto.b.22143 by Cochrane Netherlands, Wiley Online Library on [29/09/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

FIGURE 4 Correlations between MRD levels obtained by manual analyses and the AGI tool in repeated measurements. Correlations between log10 transformed MRD levels obtained by manual analysis and AGI tool with Pearson R^2. (a) Intra-expert variation (one FCS-file was repeatedly analyzed by the same expert). (b) Inter-expert variation (files were analyzed by four different laboratories).

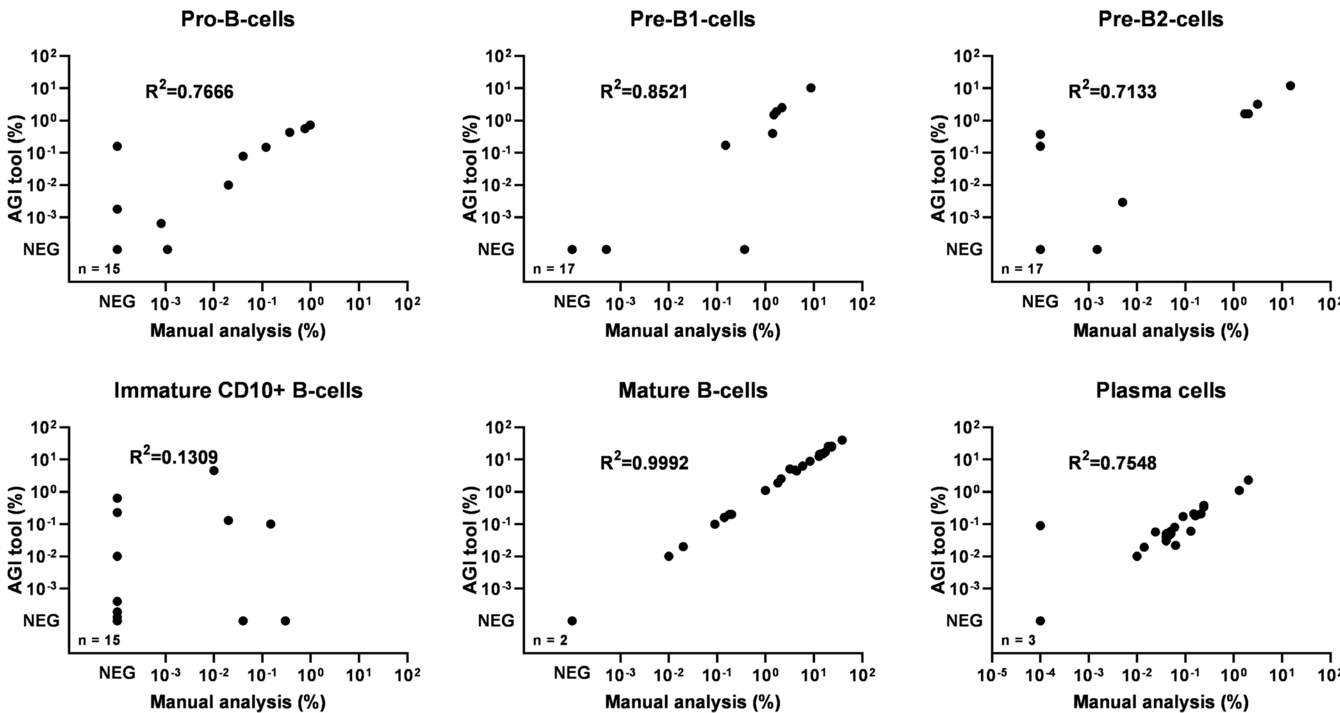


FIGURE 5 Correlations of normal bone marrow cell subsets between manual analysis and AGI tool based analysis. Correlations between percentages of each cell subset are shown with Pearson R^2 of log10 transformed data.

B-cell subsets showed a good correlation between manual and AGI tool analysis (Figure S6). However, poor correlations were found for immature CD10+ B-cells ($R^2 = 0.1309$, $p = 0.8557$), mesenchymal stem cells ($R^2 = 0.2184$, $p = 0.092$) and unspecified nucleated cells ($R^2 = 0.2717$, $p = 0.737$) (Figure S6).

4 | DISCUSSION

Most current treatment protocols for BCP-ALL are guided by MRD data. Flow cytometric MRD analysis has been standardized, but the data analysis still depends on individual expert interpretation of flow cytometry data. In this study, we aimed to develop an AGI tool for the identification of normal hematopoietic cell subsets or marked as checks by the AGI tool. These checks may represent residual leukemic cells with an aberrant immunophenotype. In addition, low quality BM aspirates or non-optimally stained samples will result in an AGI tool analysis with a large number of checks. Therefore, the number of checks may be used as an alert for sample and staining quality or for the presence of MRD. Using nBM samples, 96% of events could immediately be assigned to a normal population, and only 4% of events ended up being assigned to the checks. The AGI tool identifies normal populations by comparing, in a multidimensional space, the fluorescence intensities of the identified clusters of individual cells

15524957, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cyto.b.22143 by Cochrane Netherlands, Wiley Online Library on [29/09/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

with the fluorescence intensities of normal cells stored in a database, as previously done by EuroFlow for other antibody combinations (Flores-Montero et al., 2017, 2019). The BCP-ALL MRD reference database built here was composed of 24 normal BM FCS-files obtained from subjects at different ages, and thereby provided insight in the age-dependent cellular composition of normal BM decreasing frequencies of (precursor) B-cells and monocytes and increasing frequencies of neutrophils with increasing age. Whereas the relative distribution of populations may change with age, the immunophenotype of the cells remained similar. Therefore, it is not expected that adding more cases to the database will have a major impact on the percentage of checks, but it may improve the accuracy of the thresholds for alerts. Although some previous reports evaluated the age-dependent cellular composition of normal BM, these reports generally include only childhood or adult subjects or both considered together (Heldrup et al., 1992; Lin et al., 1998), and a few only compared individuals of different age (Lucio et al., 1999; Orfao et al., 2019). Our database, in spite of consisting of relatively low numbers, therefore, provides novel important data on age-dependent changes in cellular composition of normal bone marrow, concordant with a recently performed, comparable study (Pont et al., 2018). The relatively limited number of included cases in our study resulted from stringent quality control of available samples: only optimal samples were included, the remaining samples may still be evaluable since deviations were generally limited. This stringent quality control ensured that the database is suitable for correct identification of the normal hematopoietic cells, as confirmed by the low number of checks in the re-analysis of the database files. It should be stressed that for normal use of the AGI tool, also samples that do not undergo stringent QA, as our 174 MRD samples can be used as the aim of the AGI tool regarding identification of clusters of events to be checked by an expert relies on facilitating identification of potential technical problems, including cell carryover, in addition to MRD cells.

We validated our AGI tool with a real-life cohort of 174 BCP-ALL patient FCS-files. Of note in every MRD patient sample, the MRD events were placed by the AGI tool as checks, confirming its high sensitivity. Despite this, the first round of analysis showed a concordance of 83% between manual and AGI supported analysis. Our results showed lower concordances compared to different machine learning algorithms (DiGiuseppe et al., 2015; Fiser et al., 2012; Reiter et al., 2019; Shopsowitz et al., 2022; Wodlinger et al., 2022). However, other algorithms were generally validated with lower numbers of patients (median 123; range 20–519) and/or samples with relatively high MRD levels, and applying 0.01% as cut-off for MRD positivity. In our evaluation of the AGI tool, patient files with MRD levels down to 10^{-5} were included and also at these low MRD levels good results were obtained. In our study, the concordance between the AGI tool and manual analysis increased to 87% when 0.01% was used as cut-off, and increased to 93% using a cutoff of 0.1%. However, after review of the discordant cases by additional experts, the concordance increased to 97%, comparable to other studies. These data indicate that appropriate training is crucial for the correct interpretation of MRD data and that only after such training sufficiently robust data that allow application in a diagnostic setting can be obtained. Importantly, the AGI tool was validated with both chemotherapy and targeted therapy treated patients with comparable performance, whereas other algorithms only have been validated with chemotherapy treated patients. Altogether, these data show that the AGI tool is able to recognize the ALL cells in every patient treated with targeted therapies (and to mark them as checks) and that, in line with our previous findings (Verbeek et al., 2022), CD19 is not essential for identification of B-cells in samples analyzed with the EuroFlow BCP-ALL MRD panel. Likely, addition of extra B-cell markers, e.g. CD22 and/or CD24 (Cherian et al., 2018; Mikhailova et al., 2022) may facilitate the analysis; a 12-color EuroFlow panel including such markers is therefore being evaluated. Furthermore, in contrast to other algorithms, the AGI tool-based analysis identified normal hematopoietic subsets, as well as mesenchymal stem cells (MSCs) and endothelial cells (ECs). Both populations of BM-derived stromal cells might thereby be used as a measure of sample quality and hemodilution, as they are virtually undetectable in blood at the 10^{-5} to 10^{-6} levels. Recent studies have shown that these cells are associated with patient outcome and can be used as a prognostic marker in BCP-ALL patients (Fallati et al., 2022; Oliveira et al., 2022). CD10+ MSCs and CD34+ ECs are generally not identified in manual analysis, and the lack of experience with such cells may explain the overall lower concordance between manual and AGI supported analysis. Thus, it can be expected that the AGI tool will improve the correct identification of MSCs and ECs.

The reproducibility was determined for 10 samples which were analyzed by four different experts from different centers. Seven samples showed highly reproducible results with low %CV (<27%), whereas in three samples, one or two analyses were discordant, resulting in a 90% qualitative concordance. In our previous study (Theunissen et al., 2017), reproducibility was determined in 27 samples by six experts and 98% concordance was obtained. Detailed analysis of the cases used in this study compared to the previous study showed that the FCS-files used in the current study had lower mean MRD levels compared to the files in our previous study (0.039%–0.798%), which might explain the somewhat lower concordance. The data obtained in our accuracy study showed that discrepancies were mainly caused by different interpretations by the expert, rather than by issues caused by the AGI tool. Since the AGI tool supported MRD assessment requires knowledge of the immunophenotype of normal and malignant hematopoietic cells and still requires comprehensive training to assign checks. This manual assignment of checks leads to expert dependent interpretation of MRD assessment for AGI tool supported analysis. Discordance between manual analysis and AGI tool supported analysis could be explained by the fact that the experts have extensive experience in the manual analysis, while using the AGI tool was new for most of them; clearly one needs to get used to such other way of analysis. It should be noted that in the vast majority of analyzed cases concordant results were obtained, and that only for this limited set of FCS-files data analysis was less straight-forward.

Since the AGI tool is incorporated in the Infinicyt software, it is suitable for usage without advanced bio-informatics and without specific bio-informatics expertise. The time to run the AGI-tool is mainly

15524957, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cyto.b.22143 by Cochrane Netherlands, Wiley Online Library on [29/09/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

dependent on the quality of the computer that is used and the complexity of the FCS-file (particularly the number of events). It should be noted that the algorithm does not need manual supervision while running the AGI tool and therefore can be left unattended by the user for example overnight. For an experienced user it takes about 10–15 min to evaluate all checks and to complete the analysis, including a standardized (automated) report, which is at least as fast as manual analysis (taking about 15–20 min). A major advantage of the AGI-tool is that it results in a more standardized MRD assessment in addition to the accurate identification of the great majority of cells (>90%) in every MRD sample with the possibility to overlay each cluster of events to be checked by the expert against the normal B cell precursors identified in the same sample.

Future inclusion of reference BCP-ALL leukemia cells, in addition to normal bone marrow cells, in the BCP-ALL MRD database, may further reduce the expert-dependent interpretation and thereby, make the MRD analysis even more objective. Till then, the AGI tool will, with appropriate training, result in a more standardized MRD assessment in BCP-ALL patients.

AUTHOR CONTRIBUTIONS

Study concept and design: Martijn W. C. Verbeek, Vincent H. J. van der Velden. *Development of methodology*: Georgiana Grigore, Alberto Orfao, Jacques J. M. van Dongen, Vincent H. J. van der Velden. *Acquisition, analysis and interpretation of data*: Martijn W. C. Verbeek, Beatriz Soriano Rodríguez, Lukasz Sedek, Anna Laqua, Chiara Buracchi, Malicorne Buysse, Michaela Reiterová, Elen Oliveira, Daniela Morf, Sjoerd R. Oude Alink, Susana Barrena, Saskia Kohlscheen, Lukasz Slota, Stefan Nierkens, Mattias Hofmans, Paula Fernandez, Elaine Sobral de Costa, Ester Mejstrikova, Tomasz Szczepanski, Monika Brüggemann, Giuseppe Gaipa, Georgiana Grigore, Vincent H. J. van der Velden. *Statistical analysis*: Martijn W. C. Verbeek. *Writing and revision of the paper*: Martijn W. C. Verbeek, Vincent H. J. van der Velden. *Review and approval of the final paper*: all authors.

ACKNOWLEDGMENTS

We gratefully acknowledge all EuroFlow participants for fruitful discussions. We thank all laboratory staff of the EuroFlow laboratories for collecting data, and Marieke Bitter and Bart Lubbers for organizational and management support.

FUNDING INFORMATION

The EuroFlow Consortium received support from the FP6-2004-LIFESCIHEALTH-5 program of the European Commission (grant LSHB-CT-2006-018708) as Specific Targeted Research Project (STREP). The EuroFlow Consortium is part of the European Scientific Foundation for Hemato-Oncology (ESLHO), a Scientific Working Group (SWG) of the European Hematology Association (EHA). This study has been funded by the CB16/12/00400 (CIBERONC) and PI19/01183 grants, by Instituto de Salud Carlos III (ISCIII) and co-funded by the Fondo Europeo de Desarrollo Regional (FEDER). MR and EM were supported by the Ministry of Health of the Czech Republic, grant number NU20J-07-00028. EM was further supported by the National Institute for Cancer Research (Programme EXCELES, ID Project No. LX22NPO5102)—Funded by the European Union—Next Generation EU. MoBr was supported by the Deutsche José Carreras Leukämie-Stiftung (grants DJCLS R 15/11 and DJCLS 06R/2019). LuSe, LuSl and TS were supported by internal grant from the Medical Univeristy of Silesia: PCN-1-075/K/1/K. CB and GiGa were partially supported by Fondazione M. Tettamanti M. De Marchi ONLUS for this project.

CONFLICT OF INTEREST STATEMENT

J.J.M. van Dongen, J.A. Orfao, and V.H.J. van der Velden each report being one of the inventors on the EuroFlow-owned patent PCT/NL2010/050332 (Methods, reagents and kits for flowcytometric immunophenotyping of normal, reactive and malignant leukocytes). The Infinicyt software is based on intellectual property (IP) of some EuroFlow laboratories (University of Salamanca in Spain) and the scientific input of other EuroFlow members. All above mentioned intellectual property and related patents are licensed to Cytognos (Salamanca, ES) and BD Biosciences (San José, CA), 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. V.H.J. van der Velden reports a Laboratory Services Agreement with BD Biosciences, Cytognos and Agilent; all related fees are for the Erasmus MC. J.J.M. van Dongen and A. Orfao report an Educational Services Agreement from BD Biosciences (San José, CA) and a Scientific Advisor Agreement with Cytognos; all related fees and honoraria are for the involved university departments at Leiden University Medical Center and University of Salamanca. Georgiana Grigore is an employee of Cytognos. Monika Brüggemann reports a Laboratory Services Agreement with BD Biosciences, Cytognos and Agilent; all related fees are for the UKSH. Monika Brüggemann received personal fees from Incyte (advisory board) and Roche Pharma AG, financial support for reference diagnostics from Affimed, Amgen and Regeneron, grants and personal fees from Amgen (advisory board, speakers bureau, travel support), and personal fees from Janssen and BD (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.

ORCID

Elaine Sobral de Costa https://orcid.org/0000-0002-5340-5816
Vincent H. J. van der Velden https://orcid.org/0000-0001-9457-3763

REFERENCES

Bueno, C., Barrera, S., Bataller, A., Ortiz-Maldonado, V., Elliot, N., O'Byrne, S., Wang, G., Rovira, M., Gutierrez-Aguera, F., Trincado, J. L., Gonzalez-Gonzalez, M., Morgades, M., Sorigue, M., Barcena, P., Zanetti, S. R., Torrebadell, M., Vega-Garcia, N., Rives, S., Mallo, M., … Menendez, P. (2022). CD34+CD19-CD22+ B-cell progenitors may underlie phenotypic escape in patients treated with CD19-directed therapies. *Blood*, *140*, 38–44.

15524957, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cyto.b.22143 by Cochrane Netherlands, Wiley Online Library on [29/09/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Cherian, S., Miller, V., McCullouch, V., Dougherty, K., Fromm, J. R., & Wood, B. L. (2018). A novel flow cytometric assay for detection of residual disease in patients with B-lymphoblastic leukemia/lymphoma post anti-CD19 therapy. *Cytometry. Part B, Clinical Cytometry*, *94*, 112–120.

Comans-Bitter, W. M., de Groot, R., van den Beemd, R., Neijens, H. J., Hop, W. C., Groeneveld, K., Hooijkaas, H., & van Dongen, J. J. M. (1997). Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. *The Journal of Pediatrics*, *130*, 388–393.

DiGiuseppe, J. A., Tadmor, M. D., & Pe'er, D. (2015). Detection of minimal residual disease in B lymphoblastic leukemia using viSNE. *Cytometry. Part B, Clinical Cytometry*, *88*, 294–304.

Fallati, A., Di Marzo, N., D'Amico, G., & Dander, E. (2022). Mesenchymal stromal cells (MSCs): An ally of B-cell acute lymphoblastic leukemia (B-ALL) cells in disease maintenance and progression within the bone marrow hematopoietic niche. *Cancers (Basel)*, *14*, 14.

Fiser, K., Sieger, T., Schumich, A., Wood, B., Irving, J., Mejstrikova, E., & Dworzak, M. N. (2012). Detection and monitoring of normal and leukemic cell populations with hierarchical clustering of flow cytometry data. *Cytometry Part A*, *81*, 25–34.

Flores-Montero, J., Grigore, G., Fluxa, R., Hernandez, J., Fernandez, P., Almeida, J., Munoz, N., Bottcher, S., Sedek, L., van der Velden, V. H. J., Barrena, S., Hernandez, A., Paiva, B., Lecrevisse, Q., Lima, M., Santos, A. H., van Dongen, J. J. M., & Orfao, A. (2019). EuroFlow lymphoid screening tube (LST) data base for automated identification of blood lymphocyte subsets. *Journal of Immunological Methods*, *475*, 112662.

Flores-Montero, J., Sanoja-Flores, L., Paiva, B., Puig, N., Garcia-Sanchez, O., Bottcher, S., van der Velden, V. H. J., Perez-Moran, J. J., Vidriales, M. B., Garcia-Sanz, R., Jimenez, C., Gonzalez, M., Martinez-Lopez, J., Corral-Mateos, A., Grigore, G. E., Fluxa, R., Pontes, R., Caetano, J., Sedek, L., … Orfao, A. (2017). Next generation flow for highly sensitive and standardized detection of minimal residual disease in multiple myeloma. *Leukemia*, *31*, 2094–2103.

Gaipa, G., Basso, G., Biondi, A., & Campana, D. (2013). Detection of minimal residual disease in pediatric acute lymphoblastic leukemia. *Cytometry. Part B, Clinical Cytometry*, *84*, 359–369.

Harris, K., LaBelle, J. L., & Bishop, M. R. (2021). Current status of CAR T cell therapy for leukemias. *Current Treatment Options in Oncology*, *22*, 62.

Heldrup, J., Kalm, O., & Prellner, K. (1992). Blood T and B lymphocyte subpopulations in healthy infants and children. *Acta Paediatrica*, *81*, 125–132.

Lhermitte, L., Barreau, S., Morf, D., Fernandez, P., Grigore, G., Barrena, S., de Bie, M., Flores-Montero, J., Bruggemann, M., Mejstrikova, E., Nierkens, S., Burgos, L., Caetano, J., Gaipa, G., Buracchi, C., da Costa, E. S., Sedek, L., Szczepanski, T., Aanei, C. M., … van der Velden, V. H. J. (2021). Automated identification of leukocyte subsets improves standardization of database-guided expert-supervised diagnostic orientation in acute leukemia: A EuroFlow study. *Modern Pathology*, *34*, 59–69.

Libert, D., Yuan, C. M., Masih, K. E., Galera, P., Salem, D., Shalabi, H., Yates, B., Delbrook, C., Shern, J. F., Fry, T. J., Khan, J., Stetler-Stevenson, M., & Shah, N. N. (2020). Serial evaluation of CD19 surface expression in pediatric B-cell malignancies following CD19-targeted therapy. *Leukemia*, *34*, 3064–3069.

Lin, S. C., Chou, C. C., Tsai, M. J., Wu, K. H., Huang, M. T., Wang, L. H., & Chiang, B. L. (1998). Age-related changes in blood lymphocyte subsets of Chinese children. *Pediatric Allergy and Immunology*, *9*, 215–220.

Linskens, E., Diks, A. M., Neirinck, J., Perez-Andres, M., De Maertelaere, E., Berkowska, M. A., Kerre, T., Hofmans, M., Orfao, A., van Dongen, J. J. M., Haerynck, F., Philippe, J., & Bonroy, C. (2020). Improved standardization of flow cytometry diagnostic screening of primary immunodeficiency by software-based automated gating. *Frontiers in Immunology*, *11*, 584646.

Lu, W., Wei, Y., Cao, Y., Xiao, X., Li, Q., Lyu, H., Jiang, Y., Zhang, H., Li, X., Jiang, Y., Meng, J., Yuan, T., Zhu, H., He, X., Jin, X., Sun, R., Sui, T., Liu, K., & Zhao, M. (2021). CD19 CAR-T cell treatment conferred sustained remission in B-ALL patients with minimal residual disease. *Cancer Immunology, Immunotherapy*, *70*, 3501–3511.

Lucio, P., Parreira, A., van den Beemd, M. W., van Lochem, E. G., van Wering, E. R., Baars, E., Porwit-MacDonald, A., Bjorklund, E., Gaipa, G., Biondi, A., Orfao, A., Janossy, G., van Dongen, J. J. M., & San Miguel, J. F. (1999). Flow cytometric analysis of normal B cell differentiation: A frame of reference for the detection of minimal residual disease in precursor-B-ALL. *Leukemia*, *13*, 419–427.

Maurer-Granofszky, M., Schumich, A., Buldini, B., Gaipa, G., Kappelmayer, J., Mejstrikova, E., Karawajew, L., Rossi, J., Suzan, A. C., Agriello, E., Anastasiou-Grenzelia, T., Barcala, V., Barna, G., Batinic, D., Bourquin, J. P., Bruggemann, M., Bukowska-Strakova, K., Burnusuzov, H., Carelli, D., … Dworzak, M. N. (2021). An extensive quality control and quality assurance (QC/QA) program significantly improves inter-laboratory concordance rates of flow-cytometric minimal residual disease assessment in acute lymphoblastic leukemia: An I-BFM-FLOW-network report. *Cancers (Basel)*, *13*, 6148.

Mikhailova, E., Itov, A., Zerkalenkova, E., Roumiantseva, J., Olshanskaya, Y., Karachunskiy, A., Novichkova, G., Maschan, M., & Popov, A. (2022). B-lineage antigens that are useful to substitute CD19 for minimal residual disease monitoring in B cell precursor acute lymphoblastic leukemia after CD19 targeting. *Cytometry. Part B, Clinical Cytometry*, *102*, 353–359.

Mikhailova, E., Semchenkova, A., Illarionova, O., Kashpor, S., Brilliantova, V., Zakharova, E., Zerkalenkova, E., Zangrando, A., Bocharova, N., Shelikhova, L., Diakonova, Y., Zhogov, V., Khismatullina, R., Molostova, O., Buldini, B., Raykina, E., Larin, S., Olshanskaya, Y., Miakova, N., … Popov, A. M. (2021). Relative expansion of CD19-negative very-early normal B-cell precursors in children with acute lymphoblastic leukaemia after CD19 targeting by blinatumomab and CAR-T cell therapy: Implications for flow cytometric detection of minimal residual disease. *British Journal of Haematology*, *193*, 602–612.

Oliveira, E., Costa, E. S., Ciudad, J., Gaipa, G., Sedek, L., Barrena, S., Szczepanski, T., Buracchi, C., Silvestri, D., Siqueira, P. F. R., Mello, F. V., Torres, R. C., Oliveira, L. M. R., Fay-Neves, I. V. C., Sonneveld, E., van der Velden, V. H. J., Mejstrikova, E., Ribera, J. M., Conter, V., … Orfao, A. (2022). Bone marrow stromal cell regeneration profile in treated B-cell precursor acute lymphoblastic leukemia patients: Association with MRD status and patient outcome. *Cancers (Basel)*, *14*, 3088.

Orfao, A., Matarraz, S., Perez-Andres, M., Almeida, J., Teodosio, C., Berkowska, M. A., van Dongen, J. J. M., & EuroFlow. (2019). Immunophenotypic dissection of normal hematopoiesis. *Journal of Immunological Methods*, *475*, 112684.

Pedreira, C. E., Costa, E. S., Lecrevisse, Q., van Dongen, J. J., Orfao, A., & EuroFlow, C. (2013). Overview of clinical flow cytometry data analysis: Recent advances and future challenges. *Trends in Biotechnology*, *31*, 415–425.

Pedreira, C. E., Costa, E. S. D., Lecrevise, Q., Grigore, G., Fluxa, R., Verde, J., Hernandez, J., van Dongen, J. J. M., Orfao, A., & EuroFlow. (2019). From big flow cytometry datasets to smart diagnostic strategies: The EuroFlow approach. *Journal of Immunological Methods*, *475*, 112631.

Pont, J., Souvignet, A., Campos, L., Plesa, A., Bulabois, B., Pernollet, M., Raskovalova, T., Dumestre-Perard, C., Cesbron, J. Y., & Jacob, M. C. (2018). Accurate quantification of fourteen normal bone marrow cell subsets in infants to the elderly by flow cytometry. *Cytometry. Part B, Clinical Cytometry*, *94*, 627–636.

Reiter, M., Diem, M., Schumich, A., Maurer-Granofszky, M., Karawajew, L., Rossi, J. G., Ratei, R., Groeneveld-Krentz, S., Sajaroff, E. O., Suhendra, S., Kampel, M., & Dworzak, M. N. (2019). Automated flow cytometric MRD assessment in childhood acute B-lymphoblastic

15524957, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cyto.b.22143 by Cochrane Netherlands, Wiley Online Library on [29/09/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

leukemia using supervised machine learning. *Cytometry Part A*, *95*, 966–975.

Rodriguez, R. F., de Matos Correia, J. A., & Herrero, J. B. (2017). Method of digital information classification patent US10133962B2.

Shopsowitz, K. E., Liu, L., Setiadi, A., Al-Bakri, M., & Vercauteren, S. (2022). Machine learning optimized multiparameter radar plots for B-cell acute lymphoblastic leukemia minimal residual disease analysis. *Cytometry. Part B, Clinical Cytometry*, *102*, 342–352.

Singh, H., Manuri, P. R., Olivares, S., Dara, N., Dawson, M. J., Huls, H., Hackett, P. B., Kohn, D. B., Shpall, E. J., Champlin, R. E., & Cooper, L. J. (2008). Redirecting specificity of T-cell populations for CD19 using the sleeping beauty system. *Cancer Research*, *68*, 2961–2971.

Theunissen, P., Mejstrikova, E., Sedek, L., van der Sluijs-Gelling, A. J., Gaipa, G., Bartels, M., Sobral da Costa, E., Kotrova, M., Novakova, M., Sonneveld, E., Buracchi, C., Bonaccorso, P., Oliveira, E., Te Marvelde, J. G., Szczepanski, T., Lhermitte, L., Hrusak, O., Lecrevisse, Q., Grigore, G. E., … van der Velden, V. H. J. (2017). Standardized flow cytometry for highly sensitive MRD measurements in B-cell acute lymphoblastic leukemia. *Blood*, *129*, 347–357.

van Dongen, J. J. M., van der Velden, V. H. J., Bruggemann, M., & Orfao, A. (2015). Minimal residual disease diagnostics in acute lymphoblastic leukemia: Need for sensitive, fast, and standardized technologies. *Blood*, *125*, 3996–4009.

Verbeek, M. W. C., Buracchi, C., Laqua, A., Nierkens, S., Sedek, L., Flores-Montero, J., Hofmans, M., Sobral de Costa, E., Novakova, M., Mejstrikova, E., Barrena, S., Kohlscheen, S., Szczepanowski, M., Kulis, J., Oliveira, E., Jugooa, R., de Jong, A. X., Szczepanski, T., Philippe, J., … van Dongen, J. J. M. (2022). Flow cytometric minimal residual disease assessment in B-cell precursor acute lymphoblastic leukaemia patients treated with CD19-targeted therapies—A EuroFlow study. *British Journal of Haematology*, *197*, 76–81.

Wodlinger, M., Reiter, M., Weijler, L., Maurer-Granofszky, M., Schumich, A., Sajaroff, E. O., Groeneveld-Krentz, S., Rossi, J. G., Karawajew, L., Ratei, R., & Dworzak, M. N. (2022). Automated identification of cell populations in flow cytometry data with transformers. *Computers in Biology and Medicine*, *144*, 105314.

SUPPORTING INFORMATION

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

How to cite this article: Verbeek, M. W. C., Rodríguez, B. S., Sedek, L., Laqua, A., Buracchi, C., Buysse, M., Reiterová, M., Oliveira, E., Morf, D., Oude Alink, S. R., Barrena, S., Kohlscheen, S., Nierkens, S., Hofmans, M., Fernandez, P., de Costa, E. S., Mejstrikova, E., Szczepanski, T., Slota, L., … van der Velden, V. H. J. (2023). Minimal residual disease assessment in B-cell precursor acute lymphoblastic leukemia by semi-automated identification of normal hematopoietic cells: A EuroFlow study. *Cytometry Part B: Clinical Cytometry*, 1–12. https://doi.org/10.1002/cyto.b.22143

15524957, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cyto.b.22143 by Cochrane Netherlands, Wiley Online Library on [29/09/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License