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Approaches for detection of minimal residual disease in childhood B-cell precursor acute lymphoblastic leukemia by FlowJo and Infinicyt softwares

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Acute lymphoblastic leukemia (ALL) involves the aggressive proliferation of lymphoblasts in the bone marrow (BM), peripheral blood or extramedullary sites. "Minimal, or Measurable Residual Disease" (MRD) is a crucial prognostic factor, helping categorize the risk of relapse and guide treatment decisions. Both multiparameter flow cytometry (FC) and molecular (PCR) approaches can detect cancer cells at very low levels, beyond the capability of traditional microscopy. This study focuses on exploring a new potential within FC analysis as a primary diagnostic tool for ALL to examine the distribution of pathological cells in MRD samples stained by standardized 8-color labelling method by multidimensional softwares FlowJo® and Infinicyt® compared to conventional FC. The study included 50 patients diagnosed with B-cell precursor ALL (BCP-ALL). They were retrospectively processed and analyzed by conventional (DIVA) and multidimensional (FlowJo and Infinicyt) softwares. All of them were systematically assessed for the level of residual tumor cells in bone marrow on days with proven prognostic significance: day 15, day 33, day 78 and other time points, according to a Berlin-Frankfurt-Munster (BFM) ALL treatment protocol. There was no significant difference ($p>0.05$) between conventional (DIVA) and multidimensional (Infinicyt and FlowJo) softwares in terms of determining the percentages of residual blast cells, but FlowJo has the advantages of a semi-automated analysis tool. Data analysis based on multidimensional approach by FlowJo® and Infinicyt® softwares is able to corroborate the results of the conventional FC analysis. Additionally, it can simplify the analysis and can be used as a complementing tool for cases that require more detailed examination.

Keywords: MRD, BCP-ALL, Infinicyt, FlowJo.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children, with peaks between 2 and 5 years of age. ALL is characterized by the rapid growth of lymphoblasts in the bone marrow (BM), peripheral blood, or extramedullary locations. "Minimal, or Measurable Residual Disease" (MRD) represents a population of leukemic cells found in the bone marrow, or less frequently in the peripheral blood. These cells exhibit resistance to chemotherapy and radiation therapy, increasing the risk of relapse. They can either be residual blasts present prior to therapy or transformed secondary blasts [1,2]. Identification of MRD represents significant prognostic factor that guides therapeutic decisions, especially in pediatric cases where achieving a balance between anti-leukemic effectiveness and long-term toxicity is crucial [3–9]. During the treatment of pediatric patients with B-cell precursor ALL (BCP-ALL), the assessment of MRD involves

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periodic monitoring of bone marrow or peripheral blood samples at various intervals. Specifically, evaluations occur at the time of diagnosis, on the 8th, 15th, and 33rd days after beginning of therapy (following BFM-type protocols), before the commencement of consolidation, before reinduction, at the conclusion of intensive therapy, and during maintenance therapy based on clinical indications [10]. Currently, prognostic significance is attributed to residual cell levels ranging from 1×10^{-4} to 1×10^{-5} cells [11]. The current emphasis is on detecting MRD at the earliest possible stage using highly sensitive, specific, and reproducible methods. As a result, there is a concerted effort in developing multiparameter flow cytometry (FC) [8–11]. Both multiparameter flow cytometry (FC) and molecular techniques such as polymerase chain reaction (PCR) can identify cancer cells at exceptionally low levels, surpassing the capabilities of conventional microscopy [11–13] (Table 1).

This study aims to investigate a new potential of FC analysis as a primary diagnostic tool for ALL [14, 15].

Although less sensitive, FC has some advantages over genetic methods, namely: applicable in more than 90% of cases, highly informative with reduced expenses and quicker processing. Yet, there are several disadvantages such as: sample processing should occur within 24 hours following collection; regeneration of bone marrow post-induction could potentially result in false-positive results; interpretation becomes challenging in cases of hypocellularity, and ongoing training is necessary [11, 12, 16, 17].

The aim of this study was to investigate the distribution of pathological cells in MRD samples stained by a standardized 8-colour methodology using FlowJo and Infinicyt multidimensional software compared to conventional FC, and to investigate whether these software tools offer additional advantages in the diagnosis and follow-up of children with ALL.

MATERIALS AND METHODS

Materials

The study included 50 children with leukemia associated immune phenotypes (BCP-ALL) diagnosed and treated in the pediatric oncohematology center in University Hospital ‘‘St. George’’ in Plovdiv, Bulgaria. The research received approval from the institutional ethics committee, and individual written informed consents were obtained from the patients’ guardians. Flow cytometry procedures were conducted at the immunological research center of Plovdiv Medical University. They were systematically assessed for the level of blasts in BM on days of proven prognostic significance, according to the BFM treatment protocol: day 15, day 33, day 78 and other time points. Half of the cases belonged to the group of other time points, followed by day 78, day 33 and day 15 with the latter representing 10% of the cases (Fig. 1).

Methods

1. Conventional approach

Methods for analysis of FC data included classical manual analysis using DIVA software version 8, and sample collection was performed using FACSAria III flow cytometer (BD Biosciences, USA). Bone marrow aspirates were stained using a standardized BCP panel consisting of the following fluorochrome-conjugated antibodies: SYTO41 Pacific Blue/ CD45 BV510/ CD58 FITC/ CD10 PE/ CD34 PerCP-Cy5.5/ CD38 PE-Cy7/ CD19 APC/ CD20 APC-Cy7 (BD Biosciences, USA). All samples were processed within 24 to 48 hours of collection.

Table 1. Comparison between the sensitivity of the different methods available for MRD assessment

Sensitivity of different methods for evaluation of MRD				
Standard 4–8 colors, flow cytometry	Real-time quantitative polymerase chain reaction (RQ-PCR)	Reverse transcription polymerase chain reaction with (RT-PCR)	Digital droplet polymerase chain reaction	Next-generation sequencing
10^{-4}*	10^{-5}–10^{-6}	10^{-5}–10^{-6}	10^{-5}–10^{-6}	10^{-6}

*one blast cell in 10 000 bone marrow cells

2. Manual multidimensional approach

The third approach to analyze the 8-colour panel data was with Infinicyt multi-dimensional software using manual analysis, version 2.0.4. The developed algorithm for FC analysis using this software is thoroughly demonstrated (Fig. 2).

After exporting the FC data in fcs. format and importing it into Infinicyt, a new workspace was created. The raw data was cleaned from "debris"

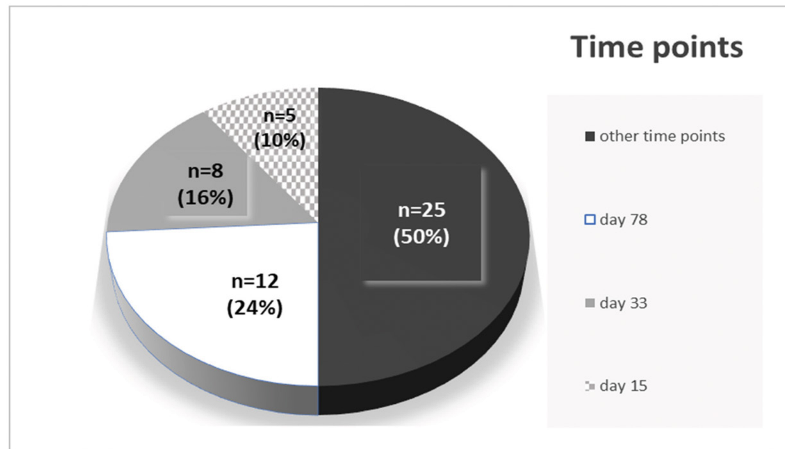

Fig. 1. Distribution of patients according to time point of flow cytometry assessment.

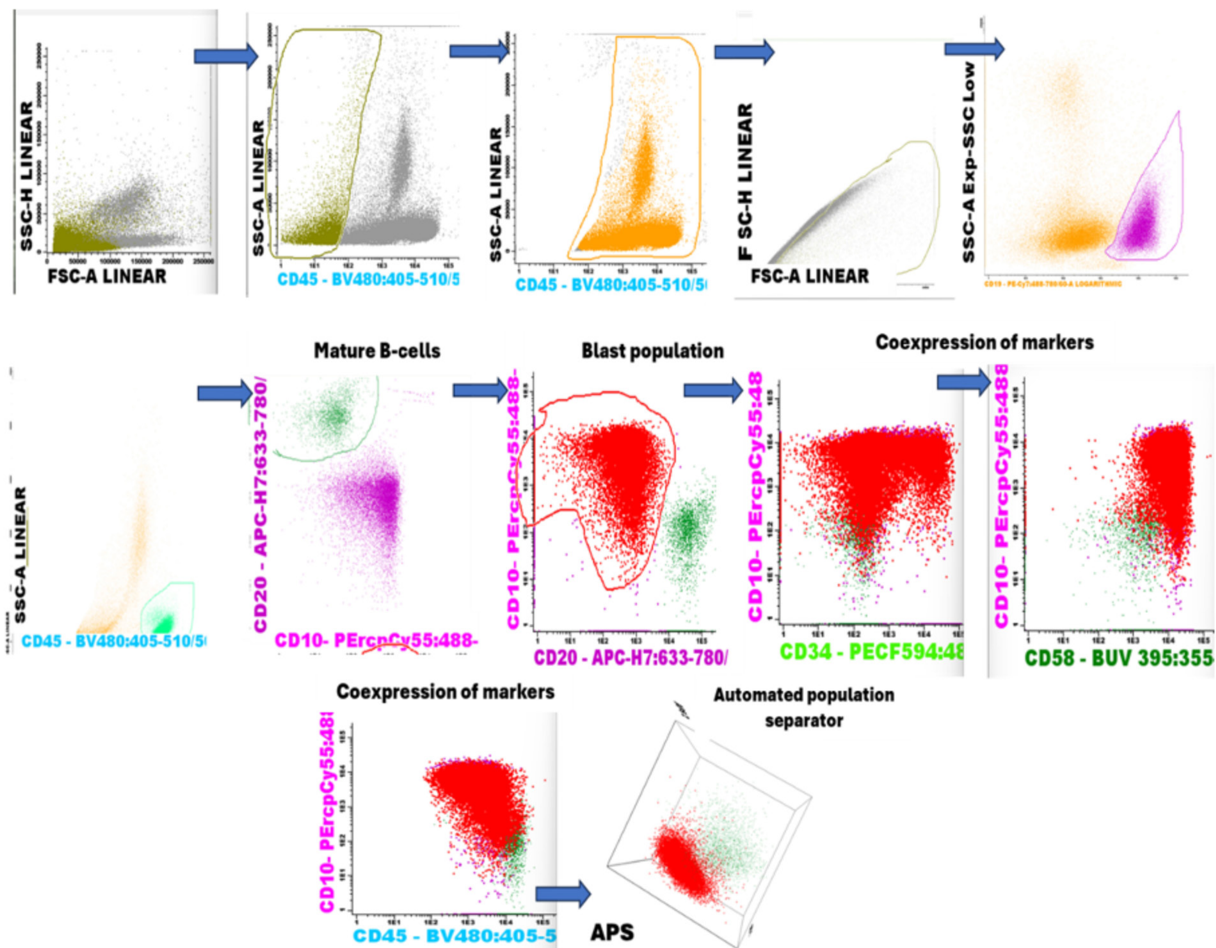

Fig. 2. Analysis algorithm with Infinicyt software.

and non-nucleated events by selecting CD45 negative events, then only nucleated CD45+ events were selected on CD45/SSC dot plot. This was followed by removal of doublets and selection of single cells only. The B-cell population was delineated based on CD19 expression, and T cells served as a negative control. Analysis of normal B-cell subpopulations was performed as well as identification of blasts based on aberrant marker expression within the B-cell population. This was followed by presentation of dot plots with co-expression of the respective markers and finally visualization using an automated cell separator (APS).

3. Semi-automated approach

The second approach was by using FlowJo semi-automated software version 10.8.1 as well as its corresponding plugins – FlowAI, flowClean, tSNE, UMAP, FlowSOM, Xshift, Phenograph, ClusterExplorer. The developed algorithm for semi-automated FC analysis is thoroughly demonstrated (Fig. 3).

A) The bivariate plot illustrates a variation in the quality of collected events. B) FlowAI plugin was applied in the first cleaning step to select only the good events (C). D) A gate based on FSC/SSC removed "debris" in the lower left corner. E) Another gate excluded doublets and selected single cells. F) CD19/SSC gating was employed to choose CD19+ events representing the B-cell population. G) FlowJo's tSNE or UMAP plugins reduced the fluorescence parameters to a bivariate plot, with individual subpopulations marked in different colors. H) Automated clustering of individual subpopulations with any of the FlowSOM, Phenograph and Xshift plugins was done. I) Subsequent visualization of phenotypic characterization using ClusterExplorer. At this stage, the cluster corresponding to the malignant population was determined by the combination of phenotypic markers.

4. Statistical Analysis

To test the equality of frequency distributions between results from conventional software and

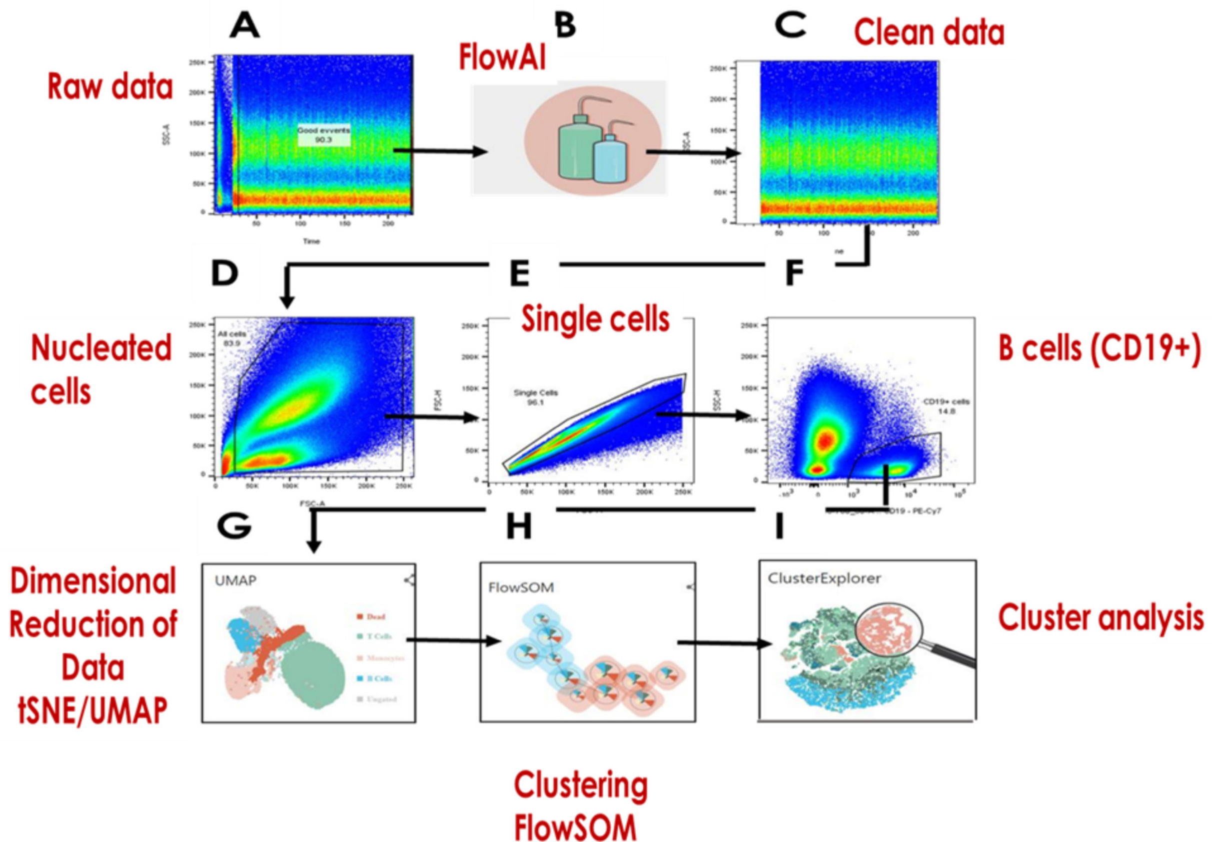

Fig. 3. Analysis algorithm with FlowJo software.

multivariate tools (Infinicyt and FlowJo), Friedman's correlated samples test was applied.

RESULTS

Friedman's test revealed no statistically significant difference ($p > 0.05$) between conventional software and multidimensional tools (Infinicyt and FlowJo) in the assessment of blast cell percentages (Fig. 4). Interestingly, the semi-automated cluster analysis tools not only identified the main cell populations but also successfully detected small subpopulations that proved challenging to discern with manual tools. This observation underscores the enhanced capability of semi-automated methods to detect cellular heterogeneity, presenting a valuable advantage in the comprehensive analysis of cell populations in comparison to conventional approaches.

Moreover, our findings provide evidence that populations appearing homogeneous on conventional two-dimensional dot plots can be further subdivided into distinct subclones through the application of semi-automated tools (Figs 5 and 6). This subclonal dissection not only gives additional information for the individual patient but also serves as a valuable strategy to mitigate the risk of subjective errors associated with manual analysis. The ability to distinguish subclonal diversity within seemingly homogeneous populations is a significant advancement, enhancing the precision of cellular characterization. This is crucial for a more comprehensive understanding of hematologic malignancies. This insight underscores the potential of semi-automated tools, such as FlowJo, to provide a more detailed perspective on cellular populations, thereby contributing to the refinement of diagnostic and therapeutic approaches for individualized patient level.

DISCUSSION

The progression of software capabilities has ushered in a transformative era in the realm of multidimensional analysis of FC data. The integration of unsupervised approaches has notably elevated the reproducibility of analyses, mitigating the reliance on manual gates through the implementation of automated identification processes for distinction of cell populations [18, 19]. The potential of automated FC approaches, particularly in the context of standardized sample analysis for hematologic malignancies, stands as a promising advancement. The ability of such tools to streamline the analysis process positions them as valuable complementary resources, particularly in cases demanding a more detailed examination of data. Furthermore, the incorporation of

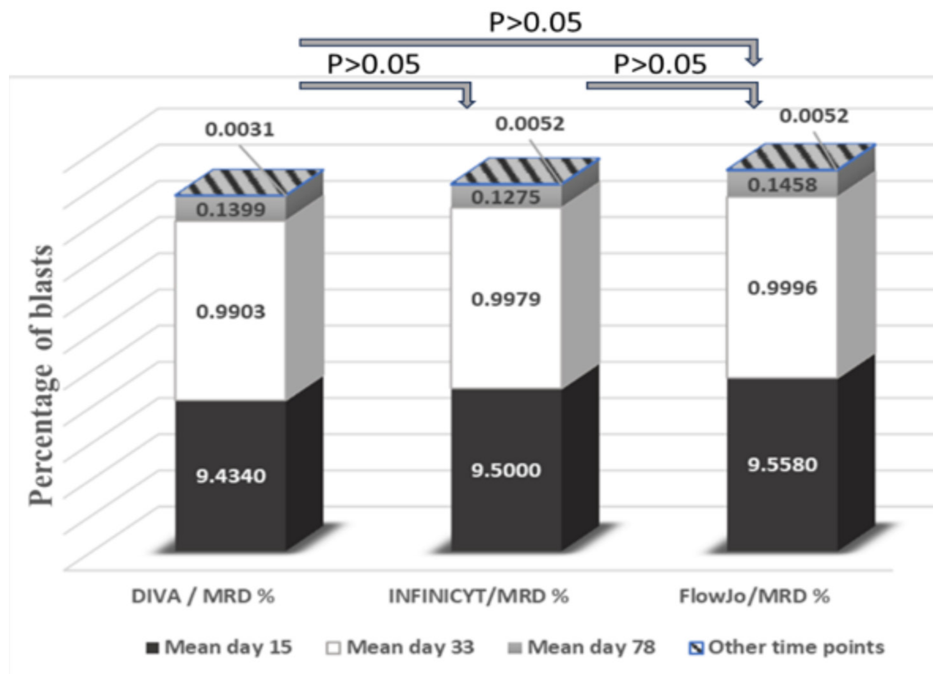

Fig. 4. Comparison between the mean values of the different time points assessed by DIVA, Infinicyt and FlowJo software.

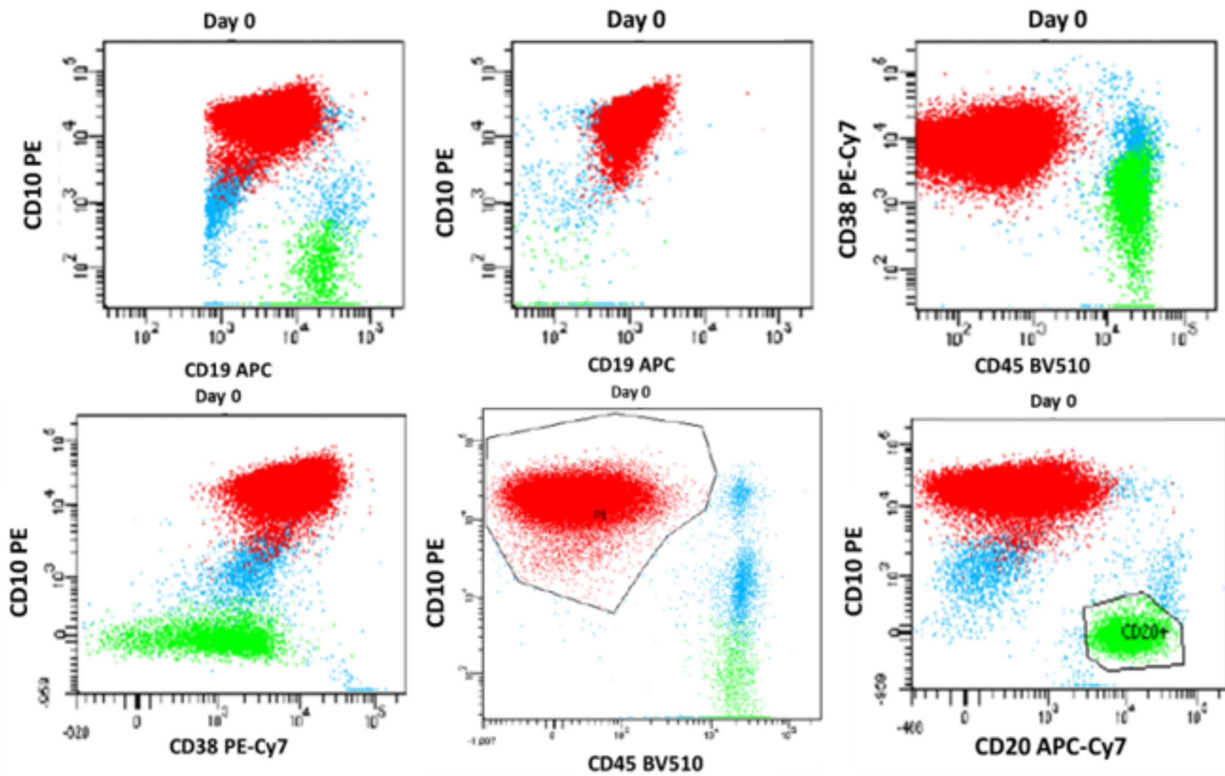

Fig. 5. Result at diagnosis (Day 0) of BCP-ALL using conventional DIVA software (93% blasts). The blast population in red has a homogeneous appearance.

semi-automated tools serves a crucial role in minimizing the risk of subjective mistakes, a potential pitfall in manual analyses. Integration of semi-automated tools emerges not only as an approach for validation of conventional results but also against interpretive errors, highlighting the importance they play in advancing the field of flow cytometry. This paradigm shift towards semi-automation not only reinforces the foundations of FC analysis but also holds promise for future developments in the understanding of cellular dynamics and heterogeneity in onco-hematological diseases [20]. Notably, the application of automated cluster analysis, underpinned by principal component analysis and regression mathematical models, extends its reach beyond the identification of major populations. It allows characterization of smaller subpopulations (e.g. FlowSOM plugin). This approach not only facilitates the identification of homogenous populations on bivariate diagrams but also unveils the capacity to detect subclones within visibly homogeneous groups [21]. The noteworthy concordance observed between results derived from conventional analyses and those employing multidimensional tools in patients with childhood ALL further attests to the efficacy and reliability of automated FC methodologies [21]. Despite these advantages, a pertinent challenge remains the requirement for standardized antibody combinations [21]. As technology continues to evolve, addressing such limitations and looking for a more comprehensive standardization will be imperative for realizing the full potential of automated FC in enhancing the understanding and management of hematologic malignancies.

CONCLUSIONS

In conclusion, the usage of semi-automated tools (FlowJo) in FC analysis marks a substantial stride towards enhancing the robustness and efficiency of conventional FC methodologies. Notably, these tools not only corroborate the findings derived from traditional FC analyses but also introduce a layer of simplification to the analytical process.

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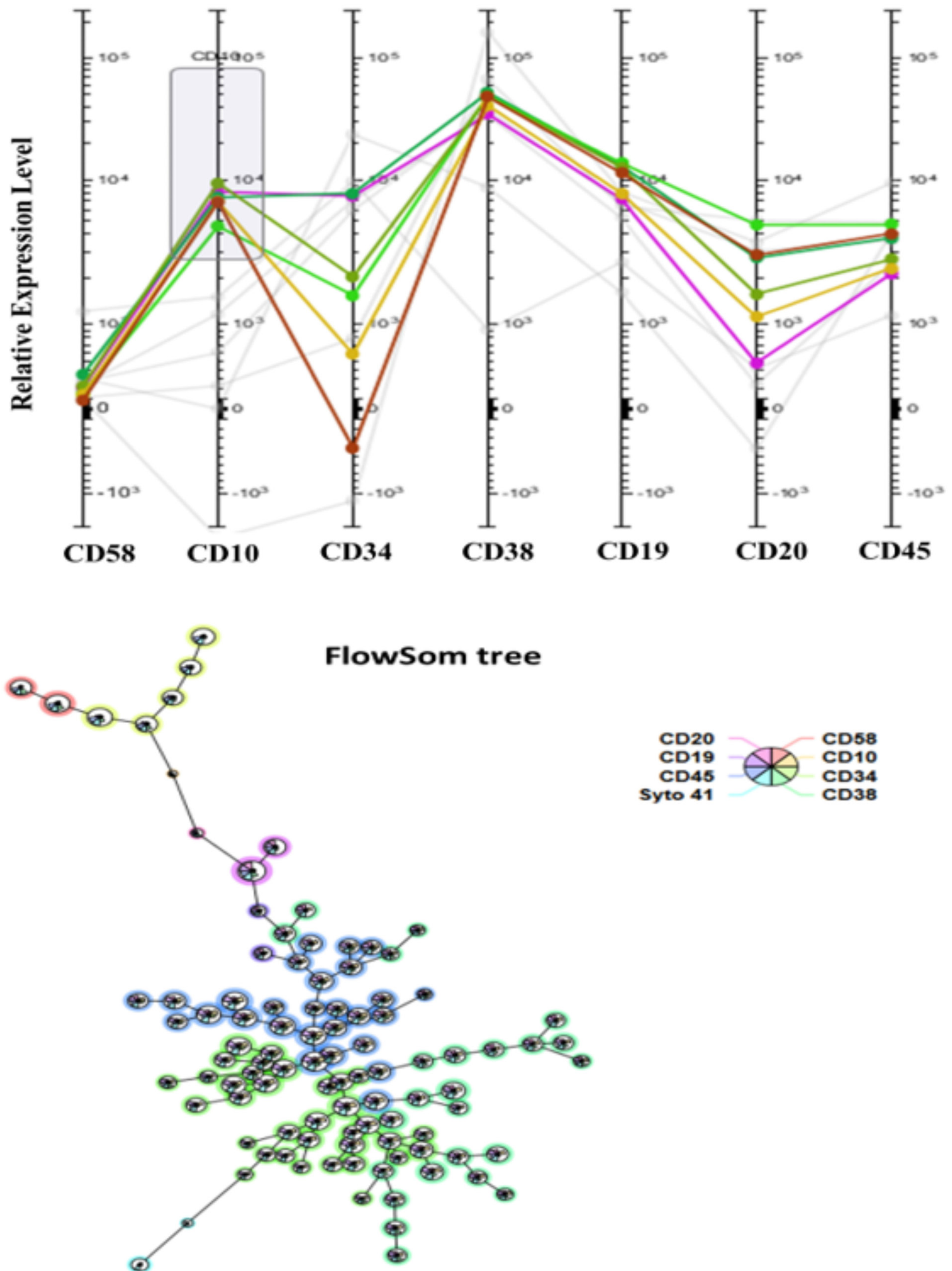

Fig. 6. Result of the same patient with BCP-ALL at diagnosis (day 0) using FlowJo software and its plugin FlowSOM (93,6% blasts). The division of the same population into several subclones is visible.

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