Implications of Minimal Residual Disease by Flow Cytometry in Pediatric Acute Leukemias

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Abstract- The most common hematological malignant diseases of the childhood are acute lymphocytic leukemia (ALL) and acute myeloid leukemia (AML). Minimal residual disease is gaining importance nowadays both for therapy efficacy follow up and relapse risk estimation. Owing to fast technological developments, minimal residual disease (MRD) can be evaluated both by molecular and flow cytometric methods, precisely. This review focuses on flow cytometry methodology and its contributions to MRD detection as well as advantages and disadvantages of flow cytometry MRD.

Keywords Pediatry, acute leukemia, flow cytometry, minimal residual disease, hematology, immunology.

I. LEUKEMIA

LEUKEMIA is a cancer type with involvement of blood or bone marrow (BM), characterized by abnormal increase of leukocytes, namely the blasts. In leukemia, due to immature cell deposition, aberrations in BM and functional defects of white blood cells (WBC) give rise to clinical symptoms. Leukemia is classified mainly into four sub-groups, first being acute or chronic leukemias, and then by affected blood cell type, as lymphoid or myeloid. Acute leukemias are usually aggressive with accumulation of early bone marrow hematopoietic progenitors, which are known as blast cells [1]. A guidebook developed by European Group for the Immunological Characterization of Leukemias (EGIL) diagnoses hematologic malignancies uniformly and sheds light on the characterization of acute leukemias, in relation to expression levels of markers. Accordingly, leukemias are divided into four groups in relation with differentiation levels and immunological properties; acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), bi-phenotypic acute leukemia and undifferentiated leukemia. ALL is divided into two groups, being as a T- or B-cell lineage. B-ALL is defined with expression of at least two early B-cell markers: CD (cluster of differentiation) 19, CD79a (mbl-1) or CD22. CD79a is expressed in cytoplasm as non-covalently bounded to cell membrane-attached immunoglobulins (Ig). CD22 is expressed intracytoplasmically during B lymphocyte maturation and on cell surface during later phases. Four categories of B-ALL are entitled, in relation with differentiation levels of blast cells, as B-I to B-IV [2, 3]. Mature form of B-ALL can be defined with expression of surface Ig or intracytoplasmic light chain. Also in these cases, unlike other B-ALL categories, expression of terminal deoxyribonucleotidyl transferase (TdT) is negative [3].

Acute Lymphoblastic Leukemia

ALL is one of the most frequent childhood diseases with malignant clonal proliferation of progenitor lymphoid cells in BM with blocked differentiation at an early stage as a consequence of somatic mutations. These abnormal cells accumulate, supersede normal hematopoietic precursors, disrupt the normal BM cell distribution and finally appear in peripheral blood (PB). ALL is a biologically heterogeneous disorder, so that morphologic, immunologic, cytogenetic, biochemical, and molecular genetic characterizations of leukemia lymphoblasts are required to establish the diagnosis or to exclude other possible causes of BM failure and, to classify ALL subtypes. This heterogeneity reflects the fact that leukemia may develop at any point of the multiple stages of normal lymphoid differentiation. Clinical parameters, immunophenotype, cytogenetic, treatment response and minimal residual disease (MRD) are among currently used factors in risk stratification and therapy determination of ALL patients [2, 4]. In many ALL protocols, days 8 and 15 of induction therapy are considered as the first checkpoints to test the in vivo sensitivity of the therapy in the individual patient. The elimination or fast reduction of the leukemia accounts for prediction of relapse-free survival [5]. The outcome for childhood ALL is being improved during the last 50 years with current full-remission rates reaching to 90%, which is possibly due to the introduction and gradual intensification of combination chemotherapies, with contemporary regimens
involving the use of 7-8 drugs, along with improvement of prognostic factors. WBC, age, gender, cytogenetic and immunophenotypic subtypes are the major initial factors determining the risk of relapse [2, 4, 5]. Standard (SR) and high risk (HR) patients have been defined by age/WBC subsets, e.g. in the classification of the National Cancer Institute: SR, age 1-9 years, and WBC <50,000/mm³; HR, age ≥10 years or WBC ≥50,000/mm³ [6]. On the other hand, a proportion of children are likely to be either over-treated or under-treated with current therapies. Thus, there is need for increased cure rates with decreased toxicity by the help of individually tailored therapies, for which, monitoring MRD gains vital importance [7].

B-ALL

B-ALL forms the majority of ALLs and is characterized by B-cell accumulation. Disease progresses with suppression of hematopoiesis and infiltration of extramedullary sites. Blasts carry a number of genetic alterations, mainly chromosomal translocations. B-cell blasts are shown to carry genetical abnormalities such as ETS related gene 6 (TEL) - RUNX1 (AML1), breakpoint cluster region protein (BCR) - Abelson murine leukemia viral oncogene homolog (ABL), E2A-Pre-B-cell leukemia transcription factor 1 (PBX1) translocations and MLL gene rearrangements [8]. B-ALL can be classified into 4 groups: Pro-B ALL, common B-ALL, pre-B ALL and mature B-ALL [3]. B cell malignancies have direct relations with normal B cell differentiation stages, as shown by immunophenotyping. Thus, B-ALL can be detected by the immunoglobulin gene rearrangement status, the pattern of expression of cellular immunoglobulin proteins and the expression of cell surface antigens [8]. The morphologic threshold for unequivocal detection of residual disease in patients with precursor B-cell acute lymphoblastic leukemia (pre-B-ALL) is generally considered to be ablast count of 5% of total nucleated cells in BM aspirate smears. First is based on variance with those expressed by normal BM cells, was applied as early as 1998 in a three color approach with CD45, systematically present in all combinations of monoclonal antibodies (moAbs). Another approach used a minimal standardized panel for B-lineage ALL, also defined for normal cells such that it becomes possible to pinpoint abnormal events. A third approach, based on the detection of leukemia associated immune phenotype (LAIP). The strategy of this group, applicable to common B-lineage ALL, was based on the detection of aberrant expression of a number of antigens on cells homogeneously defined by the combination of CD19, CD34 and CD10. The major difficulty in detecting MRD for patients with common B-ALL (CD10+) is to differentiate blast cells from hematogones, which can be quite abundant in regenerating bone marrow. The combination CD34/CD19/CD10/CD38 is quite pertinent to differentiate these cell types, based on the difference in fluorescence intensity displayed by hematogones and blasts. The value of CD58 detection for flow cytometry (FCM) MRD analysis was confirmed [9].

T-ALL

T-ALL is diagnosed with intracytoplasmic or cell-surface expression of CD3. Positive expression of CD2 or CD7 is not sufficient for T-ALL diagnose. T-ALL is classified as Pro-T-ALL (T-I) to mature T-ALL (T-IV) with respect to thymic differentiation [2, 3]. Cortical T-ALL is diagnosed with expression of CD1a, regardless of presence of membrane CD3 and other T-cell markers [3]. CD1 family members on T cells have the capacity to present lipid antigens [10]. Determination of CD1a+ T-ALL has clinical significance due to definition of better corticosteroid-responsive sub-groups. Among T-ALL, two groups are defined according to expressions of T cell receptor (TCR) chain (group a) and TCR chain (group b) together with CD3. According with some studies, two clinically and phenotypically distinct T-ALL subtypes were shown. CD10 expression can be positive in some T-ALL groups but unlike B-ALL, CD10 expression is not relevant to T-ALL classification. CD4 is not determined as a specific marker to T-cell lineage as it is expressed in monocytes and is also frequent in AML. Expression of TdT is cardinal in ALL diagnose as it is important in differentiation of ALL from mature lymphoid malignancies as large-cell lymphoma. On the other hand, expressions of other hematopoietic precursor-related markers; CD34 and HLA-DR are not taken into account in classification of the disease [3].

Acute Myeloid Leukemia

AML is a clonal disorder of hematopoietic stem cells which is characterized by inhibition of differentiation and accumulation of cells at various levels of maturity. Reduced production of healthy hematopoietic elements results with anemia, neutropenia and thrombocytopenia and gives rise to related clinical situations. AML is a heterogeneous disease, both clinically and biologically, which can be classified as M1 to M7 with different morphology, affected genes, age groups and outlooks specific to each class [11]. Blast cells may escape into PB and infiltrate cerebrospinal fluid as well as lung. High remission rates are achieved with modern chemo-therapeutic regimens nowadays, however, a large fraction of AML patients relapse, with 30-40% of young and only 10% of elderly patients being long term survivors [12]. AML’s principle feature is the variable response to therapy, which is thought to derive from different mutations, epigenetic aberrations or micro RNA expression abnormalities, producing the same morphologic disease. Analyses of nucleophosmin (NPM)-1, Fms-like tyrosine kinase (FLT)-3 and CCAAT/enhancer-binding protein alpha (CEBPA) are claimed to be important part of routine evaluation. Diagnosis of AML relies on demonstration of myeloid-lineage blasts >20% in BM or PB. Myeloid blasts typically express CD13 or CD33 surface markers. Risk stratification is done by the probability of treatment-related mortality and probability of resistance to standard therapy, for which, cytogenetics is a key predictor [13]. Although AML affects people of all ages, older people (>65 years) are claimed to be more prone to the disease, suggesting role of environmental factors on pathogenesis. Older patients also have a poor outcome. Ionizing radiation as well as DNA-damaging drugs are claimed to be disease provokers,
however, recent studies investigate multistep mutations needed to produce AML. Identification of specific gene abnormalities has important effects on improvement of prognostic allocation of patients with AML. Risk category estimation as HR / LR is very critical for determination of proper therapy. On the other hand, if early identification of high-risk patients is missed, timely use of intensive treatments may be hampered.

**Bi-phenotypic acute leukemia:** Five percent of acute leukemia cases were observed to be difficult to classify with routine techniques, due to co-expression of some myeloid and lymphoid antigens in the same cells. This type of leukemia is known as bi-phenotypic or mixed-lineage acute leukemia which is a rare type of leukemias. Accumulating data reports the poor prognosis of bi-phenotypic acute leukemia [14].

**Acute undifferentiated leukemia:** Acute undifferentiated leukemia (AUL) is the type in which the predominating cell is so immature to be classified. Leukemias in this category do not have a phenotype of single lineage allowing simple classification. Blast cannot be classified into myeloid of lymphoid lineages due to lack of expression of lineage-specific markers. The cells should be monitored by FCM for uncommon precursors like natural killer (NK) cells, plasmacytoid dendritic cells and basophiles as well as non-hematopoietic tumors. With the absence of these uncommon lineage-specific markers, diagnosis of AUL could be possible [15, 16].

### II. Minimal Residual Disease

MRD is the name attributed to the very low number of cells remaining in the patient during or after the treatment; namely in the remission period. MRD is the major reason for relapse of leukemia and is detected by molecular methods as a golden standard, but due to development of technology, FCM for MRD determination are getting developed day-by-day. MRD detection has several important roles; investigating the presence of tumor cells after therapy, determining efficacy of treatment, monitoring remission status of the patient and forecasting the possibility of a relapse [17].

In a great number of patients with leukemia, MRD can be detected by casual microscopy techniques in full remission [9]. MRD detected in early phases of therapy, independent of classical prognostic indicators like age and WBC count, immunophenotype and cytogenetic analysis is shown to provide prognostic information [7]. The patient is accepted to be in morphological remission with blast cell rate below 5% of total BM cell population. Chemotherapy-resistant blast cells in BM surviving in small amounts are shown to have capacity to trigger future relapses [18].

Following chemotherapy or stem-cell transplantation or future relapses, MRD detected by FCM or polymerase chain reaction (PCR), is of vital importance in most leukemia sub-types. These experiments are utilized in clinical studies which aim to propose new approaches to optimize therapy and also monitor its efficiency. MRD detected by FCM or PCR is accepted as an important prognostic factor in ALL [9]. There are two basic methods for detection of MRD in childhood ALL, molecular analysis of B and T-cell receptor gene rearrangements and FCM analysis of aberrant immune phenotypes [7, 19], both of which are predictive for the outcome [7]. With both methods, it is possible to detect a single leukemic cell in 10,000 or more normal cells, increasing sensitivity a minimum of 100 folds, when compared to conventional light microscopy.

Blast count in the PB on day 8, or in the BM on day 15, have been widely used to deliver risk-directed therapy. Recently, most groups explored the feasibility of MRD detection [20], and this is now widely applied to assign patients different risk categories during first phase of treatment [20-23].

Identification of prognostic factors is critical in determination of therapy for sub-groups of patients to achieve a high cure rate with minimal toxicity. Another important goal of risk stratification is to define patients with a very high risk of relapse, who may benefit from allogeneic hematopoietic stem cell transplantation following first complete remission. Large study groups defined risk classification (low, intermediate and high risk) with regards to various risk factors [2-4, 24-27].

**MRD Detection by Flow Cytometry**

FCM is a laser-based technology frequently used in science of immunology as well as in hematology, molecular biology, pathology and biology. With moAbs, FCM has allowed identification and quantification of subsets of the major lymphocyte populations and even further sub-divisions that differ in biologic function, maturation stage, and activation. FCM is becoming established in the routine evaluation of the immune system in diseases where immune disorders may play a role, including immune deficiency diseases, and particularly acquired immune deficiencies [28]. By multi-parameter FCM, oncogenesis, apoptosis or cell cycle of a defined cell population and also cellular events like DNA content can be studied. One major application of FCM is cell immunophenotyping [29] which helps to distinguish between healthy and diseased cells, that can be used to aid the diagnosis and also monitoring of myelomas, lymphomas, leukemias, immunodeficiencies as well as infections [30, 31]. In addition to utilization in medicine, FCM has applications in veterinary medicine, microbiology and fisheries [32-35]. FCM has a unique property which is of vital importance in MRD detection; the ability to distinguish viable cells from BM debris and dead cells [36]. With the great evaluation in recent years, FCM is now accessible for MRD detection, by monitoring of malignant clones and most importantly by exploring viable cells [9]. FCM detection of MRD is based on differential diagnosis of leukemic cells from normal, healthy cells by expressions of aberrant antigens by them. FCM is a rapid, convenient, and generally applicable technique for detecting MRD, defined as disease undetectable by morphologic examination, can be more than 100 times more sensitive than morphologic examination [37].

Immunophenotyping aiming the identification of pathologic cells and phenotype characterization is based on specific pattern-identification of surface as well as intracellular antigen expressions in unique cell populations. Such phenotypic aberrations are important for identification of pathologic cells among normal cells [38]. Immunophenotypic features of ALL cells vary from normal hematopoietic cells, including hematogones and activated lymphocytes. These variations are
known as LAIPs, which can be grouped into three main categories and they are principal elements of MRD monitoring by multi-parametric FCM. The first of these are immunophenotypes that are expressed during normal development but are limited to cells in certain tissues. This group is exemplified by the immunophenotypic features of T-lineage ALL cells, which are expressed only by a subset of thymocytes and can never be found outside the thymus. The second group of LAIP is constituted by the expression of fusion proteins derived from chromosomal breakpoints, such as BCR-ABL1, ETS variant gene 6 (ETV6) - Runt-related transcription factor 1 (RUNX1), or transcription factor 3 (TCF3) - Pre-B-cell leukemia transcription factor 1 (PBX1). These markers are leukemia specific, but their use has been limited by the lack of suitable antibodies for their reliable FCM analysis. The third type of LAIP is represented by markers normally expressed during lymphohematopoiesis but found in abnormal combinations in leukemic cells; namely asynchronous or aberrant. They offer the most widely available option to monitor MRD in ALL by FCM, and the only one that can currently be used to track MRD in B-lineage ALL. The use of this approach requires a deep understanding of the immunophenotypes expressed by normal hematopoietic cells, not only during steady-state conditions, but also during chemotherapy and active regeneration [39].

The challenge in MRD detection is distinguishing residual leukemic cells from non-malignant cells, including stages where regenerating BM may contain earlier maturation forms than normal samples at a steady stage of hematopoiesis. This presupposes both excellent immunophenotypic knowledge of both the malignant clone and the normal BM. Leukemic cells may have similarity to normal cells with blocked differentiation and are recognized as such in FCM diagnosis by increased numbers compared to the scarcity of normal cells expressing the same immunophenotype [9].

The association of side scatter (SSC), a parameter defining cell granularity, with an immunologic marker is the best option for blast cell identification. The best marker for this aim is an antigen expressed on all leukocytes, differentially expressed in major leukocyte subpopulations, being more densely expressed in lymphocytes and monocytes than in granulocytes; CD45. Blast cell-expression of CD45 is typically lower in leukemic lymphocytes when compared to healthy ones. For increasing the power of this approach, additional lineage-associated antigens can be used, especially in BM samples having normal residual precursors. Assessment of antigen expression is based on the use of a combination of fluorochrome conjugated moAbs and direct immunofluorescence techniques [38]. In other words, the need to use CD45 is for discrimination of cell subsets and most notably the immature/blastic cells with low SSC and low CD45 expression patterns. Utilization of this approach in a first gating for the selection of MRD within maturing cells in BM is becoming increasingly common. There are antibody combinations for ALL-MRD detection in FCM. One example was estimated as CD19/CD34/CD10; CD13/CD33/CD34, CD13/CD33/CD117 and CD13/CD34/CD117. These immunophenotypic features should be assessed at diagnosis, in order to retain information as to the specific characteristics of a given leukemic clone in terms of co-expression and fluorescence intensity of differentiation antigens. The subset of leukemic cells which persists will retain a clonal quality and these residual cells appear as a tight cluster of cells still with a frozen immunophenotype among maturing cells in FCM studies. It is commonly admitted that a cluster of between 10 and 100 MRD cells should be identified in a given sample to ensure that MRD cells have been seen. Thus, in order to achieve a sensitivity between 10^{-4} to 10^{-5}, approximately 10^5 to 10^6 leukocytes must be screened, stressing again the value of assessing MRD in samples with normalized cell counts [9]. The most important limitation for FCM MRD analysis is highly operator-dependent results and possible erroneous interpretations with respect to lack of knowledge [40].

Preparation of Samples for FCM MRD Detection

The sources of materials for MRD detection are PB and BM. Studies investigating utilization of these sources reported variable results. In general, for ALL, PB samples are claimed to be suitable for estimating phenotype and identifying blast in a day 0 sample in ALL, but MRD following induction therapy should be monitored in BM samples. In patients from whom BM samples are not available, it is possible to determine LAIPs in PB with low blast counts, which is an option solely available to FCM MRD [7]. When AML is the case, studies report similar results obtained from both PB and BM in MRD detection [41]. There are two major methods for sample preparation: ficoll enrichment and erythrocyte lysis. Ficoll enrichment was the standard procedure for sample preparation from ALL patients [42]. ALL MRD is often expressed as a proportion of mononuclear cells rather than as a proportion of leukocytes, as recommended for AML. However, density gradient centrifugation may lead to loss of the MRD cells in AML studies. Therefore, in AML MRD studies, total cellular analysis following red cell lysis, especially in no-wash procedures, is the suggested method which provides the most relevant clinical picture and this method is preferred also for ALL patients nowadays [9]. With direct procedures, possible alterations in antigen expression levels, and the work time as well as the work load are reduced. Whole blood sample preparation consists of a short period of sample incubation (typically 15-20 minutes in the dark at room temperature) with fluorochrome-conjugated moAb, which is followed by lysis of erythrocytes. Analyses must be performed on the total sample, only excluding debris/platelets, by light scatter [38]. For quality control evaluations for MRD measurements, utilization of a live-cell-permeant nucleated-cell dye and acquisition of at least 300,000 events per moAb combination from 750,000 stained cells is of vital importance [43].

Detection of B-ALL MRD

Assessment of B-ALL MRD by FCM is considered to be based on the reproducibility of the leukemia immunophenotype detected at diagnosis. In MRD detection, expression levels of 7 different antigens in BM samples of B cell precursor ALL (BCP-ALL) are determined during diagnosis and are compared at different time points of remission induction therapy. Related findings showed diminished levels of CD10 and CD34,
increased levels of CD20, CD45RA and CD11a, while CD58 was unchanged. Leukemic cells were completely discriminated from normal residual B cells with these findings [44, 45]. According to BFM protocol, blasts demonstrated during MRD evaluation were CD10-high and CD45-low [44]. FCM detection of CD58 (lymphocyte function-associated antigen-3 (LFA-3), an Ig super family member expressed by hematopoietic and non-hematopoietic cells) is shown to be an attractive tool for MRD detection in childhood B-ALL. CD58 has been shown to be over-expressed in leukemic blasts when compared to their normal counterparts; CD19\(^+\)CD10\(^-\) B-cell progenitors. This feature allowed the detection of MRD in BM samples from children affected by ALL in clinical remission. The results obtained from samples at week 12 suggest that MRD detection utilizing CD58 performs very well even in the presence of a high proportion of immature B cells [45] (Figure 1, shows our data on MRD detection of low- and high-risk B-ALL cases). Studies to differentiate hematogones from blasts gain importance in recent years. However, as it is difficult to distinguish normal immature cells and leukemic cells in some patients owing to the absence of specific and informative markers, normal lymphoid progenitors may be mis-interpreted as MRD. Thus, the accuracy and reliability of FCM based MRD measurement may be limited at BM regeneration phase [42]. As an example, the expression of CD81, a tetraspin family member integral membrane surface protein, forming a multi-molecular complex with CD19 and CD21 and responsible from transduction signals in B-cells, is found to be pale aberrantly in cases with Pre-B ALL, which is claimed to help differentiation of hematogones from Pre-B-ALL cells [46]. These findings suggested the usefulness of CD81 expression in distinguishing pre-B-ALL cells from hematogones [46]. Decreased CD81 expression is suggested to be a sensitive and specific marker for residual pre-B-ALL, even in a background of hematogones, which makes CD81 a useful addition to a MRD detection panel [37]. ZAP-70 (ζ-associated protein 70 kDa) is a protein tyrosine kinase (PTK), which is critical for signal transduction in lymphocytes following antigen recognition. ZAP-70 expression may hold prognostic value for pre-B ALL and raises the prospect of a novel therapeutic target [47]. With the aim of identifying new markers for ALL-MRD detection, genome-wide gene expression of lymphoblasts from patients with newly diagnosed childhood ALL was compared to that of normal CD19\(^+\)CD10\(^-\) B-cell progenitors. Expression of genes differentially expressed by > 3-fold in ALL cases was tested by FCM in B-lineage ALL and non-leukemic BM samples, including samples containing hematogones. CD44, B-cell lymphoma 2 (BCL2), heat shock protein beta 1 (HSPB1), CD73, CD24, CD123, CD72, CD86, CD200, CD79b, CD164, CD304, CD97, CD102, CD99, CD300a, CD130, Pre-B-cell leukemia transcription factor 1 (PBX1), cadherin associated protein 1 alpha (CTNNA1), integrin beta-7 (ITGB7), CD69, CD49f were differentially expressed in ALL cases; expression of these markers were associated with the presence of genetic abnormalities. When incorporated in 6-color combinations, the new markers were claimed to have capacity to detect 1 leukemic cell among 10\(^5\) BM cells [48].

Detection of T-ALL MRD

Early T-ALL precursors are present in bone marrows of T-ALL’s and these precursors form thymocyte subsets which migrate to thymus from bone marrow. They are hypothesized to be differentiated from hematopoietic stem cells due to their multi-lineage differentiation potentials. They have discriminative phenotypes as CD1\(^+\), CD8\(^-\) and CD5\(^{dim}\) together with stem cell- and myeloid-markers [49]. MRD can be detected from BM samples with TdT, intracellular CD3, CD7 and CD99 expressions, which are specific to blast cell populations (Figure 2, shows our data on MRD detection of low- and high-risk T-ALL cases). CD99 was shown to be a valid complement to TdT in quantifying T-ALL MRD. CD99 (E2, MIC2) is a human 32-kD transmembrane sialoglycoprotein expressed on many hematopoietic and non-hematopoietic cell types. It is involved in cellular adhesion and migration as well as in survival regulation. Initially, CD99 was described as a thymus-leukemia-associated antigen. Later on, other malignancies, such as Ewing’s tumor (ET), AML, and lymphoblastic lymphomas, were found to be CD99 positive [50]. CD99 over-expression is either non-existent or extremely rare on T cells elsewhere than thymus. CD99 is claimed to be a highly informative marker for MRD detection in T-ALL, bearing the advantage of surface expression in contrast to TdT (CD99/CD7/CD5/sCD3 surface only) [51]. Discrimination of
NK cells from blasts is a highly important issue. The blastic cells present in different malignant hematological disorders might, however, represent an overlapping population with the lymphocytes, NK-cells, monocytes, and nucleated erythroid elements on the Forward scatter (FSC) vs. SSC histogram, making it difficult or impossible to separate them from the residual reactive cells. This will add false mixed immunophenotypic property to the blastic cells [52]. The immunophenotypes of NK series leukemia were antigens from hematopoietic stem cells to T/NK progenitor cells with myeloid antigen positive, and through NK progenitors to mature NK cells. The immunophenotypes of heterogeneous NK leukemia cells are different, that should be carefully distinguished [53]. Phenotypic FCM analysis was thus found to be useful for differential diagnosis of T-cell and NK-cell lymphomas [54]. Some cases of T-ALL express NK markers like CD56 and CD16, and the presence of CD56/CD16 was associated with distinct clinical features and expression of cytotoxic molecules in the blasts [55].

is claimed to be between $10^{-4}$ and $10^{-5}$ [13]. According to recent studies, LAIPs can be detected in approximately 95% of newly diagnosed AML patients with a sensitivity of 0.1-0.01% [40]. The studies also suggest PB as a source for MRD studies in AML patients. The levels of MRD after induction and consolidation was shown to be in concordance with BM and PB and MRD >0.015% was shown to be associated with a subsequent relapse [36]. As clinics move toward a more personalized approach for treatment of AML, it will be essential to establish standardized MRD analysis methods. A study reported the most frequent aberrations in AML observed as CD33+CD77+CD11b+ (± CD34+) and CD34+/CD11b+ (≠CD117+) [9]. It is generally accepted that in AML the presence of myeloid cells of clearly immature phenotype in PB is aberrant, with SSC/low CD45. Monitoring of AML-MRD can be defined by a combination of 4 to 5 surface markers, possibly associated with flow cytometric physical abnormalities that normally are not present in a healthy BM. The most frequent aberrations can be grouped as: simultaneous expression of early and late markers (asynchronous antigen expression), expression of lymphoid-associated markers on myeloid blasts (lineage infidelity), over-expression of antigens, abnormal FSC/SSC properties of myeloid blast cells and lack of myeloid-specific antigens on myeloid blasts [9].

**Pitfalls in MRD detection of ALL by FCM**

Early detection of BM MRD on day 15 by FCM is feasible and has a powerful prognostic effect, an early precursor of relapse and can be applicable to mostly all patients. PCR-MRD detection can be helpful in later phases and it may be possible to determine patient-tailored therapies by this way [20]. Determination of MRD by FCM is based on reducibility of leukemic immunophenotyping defined during diagnose.

FCM has methodological advantages due to technical simplicity, applicability and time/cost effectiveness [19]. In comparison with molecular methods, advantages of FCM MRD are obtaining results hours after sampling and being inexpensive due to low costs [18, 20]. According to data from UK, cost of FCM MRD is 70% of molecular MRD. Increase of used fluorescent colors from 4 to 6 may increase costs as well as rapidity. Another big advantage is the exclusion possibility of apoptotic blasts by FCM, which is a reason for false positivity of PCR results. In addition to this, in cases whose BM sampling is impossible and thus not suitable for molecular MRD methods, it is possible to detect LAIPs from PB samples in which blast counts are low [7]. Disadvantages include possible false-negatives due to immunophenotypic modulation induced by drug therapy or due to the selection of a minor sub-population of blasts. To circumvent this, standardized protocols does not rely on the over expression of CD10 as a sole marker, avoids rigid gating and recommends the tracking of at least two LAIPs per patient. FCM MRD analysis with four colors cannot routinely reach the same level of sensitivity as molecular methodologies but attains the level of sensitivity required for risk stratification and has been shown to be predictive of outcome. One important observation is that samples with highly discordant results and in which molecular MRD was significantly higher than FCM, were classified as
CD10 negative/weak. The reduced CD10 antigen expression can make gating analyses more difficult and further optimization of the standardized method may be necessary for this minor subgroup of patients [7].

The increasing application of FCM was soon reflected in ALL studies. A number of investigators studied the correlation between FCM data and results obtained with PCR analyses, showing good correlation and emphasizing the fact that FCM could be used for more patients. Later on, the increasing sophistication of FCM led to more and more pertinent studies. A study demonstrated a simplified assay based on a three-color combination (CD19/CD10/CD34), which was highly significant for predicting relapse in childhood B-lineage ALL, when applied at the post induction time point [9, 56]. Following the investigation of TEL-AML1 translocation using the reverse transcription-PCR as prognostic indicator in patients with ALL by Ozbek et al, MRD with FCM was initiated in Istanbul University, Institute of Experimental Medicine (DETAE), Department of Immunology with cooperation of AIEOP-BFM partner laboratory Vienna (St Anna Children’s Cancer Research Institute) at September 2009 [57]. After training with iBFM Flow twinning program, our center, being the first in Turkey, received certificates of proficiency for B-ALL on 30.08.2011 and for T-ALL on 26.08.2013 and is able to evaluate cases by itself (Türkiye Klinikleri J Med Sci doi 10.5336/medsci/34758).

III. CONCLUSIONS

Although being less-standardized, FCM is less expensive, faster and can give information from more patients in less time. As yet, FCM-MRD analyses have largely been performed in single reference laboratories but to be incorporated into larger national and international trials, a quality assured, standardized method which can be performed in a multi-center setting might be preferable. In general, MRD detection from BM samples is an important prognostic factor for childhood leukemia follow up, but the laboratories should utilize properly-calibrated FCM, well-written and strongly applicable standard operation protocols as well as well-educated and frequently updated personal is needed.

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