



# Monitoring Measurable/Minimal Residual Disease in Acute Myeloid Leukemia: Multiparametric Flow Cytometry-Based Approach

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## Abstract

Measurable/minimal residual disease (MRD) status is the most relevant predictor of clinical outcome in hematolymphoid neoplasms, including acute myeloid leukemia (AML). In contrast to acute lymphoblastic leukemia, multiple myeloma, or chronic lymphocytic leukemia, etc., AML is a widely heterogeneous neoplasm with poor clinical outcomes. Multicolor flow cytometry (MFC) is a powerful technology with high sensitivity, rapid results, cost-effectiveness, and easy availability. It is routinely used for diagnosing and MRD monitoring in many hematological neoplasms. However, MFC-based MRD monitoring in AML is complex and challenging. It requires a refined approach, a wide panel of markers, and adequate training and experience. This review focuses on the panel design, processing details, template design, analysis approach, and recent updates in MFC-based MRD monitoring in AML. It further describes the normal distribution and maturation patterns of various sublineages among hematological progenitors and their utility in studying AML MRD.

## Keywords

- ▶ flow cytometry
- ▶ minimal residual disease
- ▶ acute myeloid leukemia

## Introduction

Acute myeloid leukemia (AML) is a group of genetically heterogeneous disorders with unpredictable clinical outcomes. The diagnosis of AML is a complex process that requires the evaluation of multiple factors, including morphology, immunophenotype, and underlying genetics. AML largely affects older adults and has a poor prognosis, with only 35 to 40% of patients younger than 60 years and 5 to 15% of patients older than 60 years achieving long-term remissions.<sup>1–4</sup>

Intensive chemotherapy has been the standard treatment for AML for many years, sometimes followed by allogeneic stem cell transplantation. The basis of improved outcomes in modern cancer therapy is risk-adopted therapeutic protocols where treatment intensity is modified based on the favorable risk versus high-risk disease. Genetic abnormalities form the basis for pretreatment prognostication in AML; however, they do not apply to some patients.<sup>5</sup> A robust factor for

predicting disease-free survival (DFS) is an initial response to therapy, that is, complete remission (CR).<sup>6–10</sup> Morphologic CR, that is, less than 5% blasts on cytomorphology in bone marrow (BM) aspirate, has been used as the clinical endpoint for evaluating chemotherapy efficacy. The cytomorphologic evaluation of CR has limitations, such as imprecision in quantifying myeloblasts using light microscopy counting up to 500 nucleated BM cells in a regenerating marrow and intra-/interobserver variability in identifying myeloblasts.<sup>11,12</sup> In the last few years, it has been proved that the traditional method of evaluating CR through monitoring the percentage of blasts in the BM and peripheral blood using microscopic examination lacks the sensitivity to detect the leukemic blasts present at low levels.<sup>11–13</sup> Such low-level residual disease can be detected by only sensitive ancillary techniques such as flow cytometry or molecular methods (quantitative polymerase chain reaction [qPCR] and next-

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generation sequencing [NGS]) and is known as minimal/measurable residual disease (MRD).<sup>14–16</sup> Several studies suggest that identifying residual disease at levels far below the conventional 5% blast threshold based on morphological analysis is a crucial tool in refining the approach to risk classification in leukemia.<sup>17–22</sup> MRD refers to the detection of leukemia cells at levels as low as 1 in 10,000 to 1 in 1,000,000 white blood cells (WBCs), which is significantly lower than the 1 in 20 thresholds in morphology-based diagnosis.<sup>23–25</sup> Studies have shown that traditional morphologic CR may not effectively monitor initial therapeutic response in AML.<sup>11–13,26,27</sup> Several studies have demonstrated that MRD is a powerful predictor of DFS in AML.<sup>14–16</sup> It provides valuable information on disease response to the initial course of intensive therapy and helps predict outcomes in a given patient. Thus, it allows reliable monitoring of treatment effectiveness, identifying patients at high risk of relapse, and making informed decisions about the need for additional therapeutic intervention wherever possible.<sup>17,22,25,28,29</sup>

Over the past 20 years, there have been advancements in the methods for detecting AML MRD, such as multiparametric flow cytometry (MFC), qPCR, and NGS-based MRD methods.<sup>23,25,29–36</sup> MFC is considered one of the most sensitive and specific methods for MRD detection in AML. This technology can detect even small numbers of residual leukemic cells among thousands of other hematopoietic cells, including normal myeloid progenitors, based on differences in cell surface markers. Major advantages of MFC-MRD over other techniques include easy availability, wider applicability (>90% of AML), cost-effectiveness, and rapid turnaround time (TAT).<sup>30,35,37,38</sup> Although theoretically, molecular methods can have higher sensitivity, due to limited applicability and the expensive nature of technology, practically, MFC-MRD has higher MRD detection sensitivity in most cases except in AML where MRD is performed by qPCR for NPM1 mutations.<sup>39,40</sup> The sensitivity of MFC-MRD can be reached beyond 0.01% in AML cases with definitive immunophenotypic aberrancies and in identifying cells with immunophenotype of leukemic stem cells (LSC). MFC-MRD not only can track the original clone of residual disease but also can easily identify new clones based on different-from-normal (DfN) approaches, which can be missed using molecular techniques based on targeted NGS or qPCR.<sup>36,39</sup> Another valuable advantage of MFC-MRD is that it allows easy assessment of hemodilution and, thus, the quality of marrow being assessed for MRD detection. However, a major limitation of this method is that it is observer dependent and needs expertise with adequate experience and a standardized approach.<sup>30</sup> Another limitation of MFC-MRD is that it may not detect mature differentiating myeloid cells with genomic aberrancies, such as BCR::ABL1 in the CML-chronic phase or mature myeloid cells or monocytes with NPM1 mutations or PML::RARA in cases of acute promyelocytic leukemia.<sup>36,40</sup> Recent studies have emphasized that incorporating AML MRD by using both MFC- and NGS-based MRD provides better prediction of clinical outcomes in AML.<sup>3,24,29,32,36,41,42</sup> This review is focused on an approach to develop antibody panels, standardization, template designing, and data analysis for MFC-MRD in AML.

## Discussion

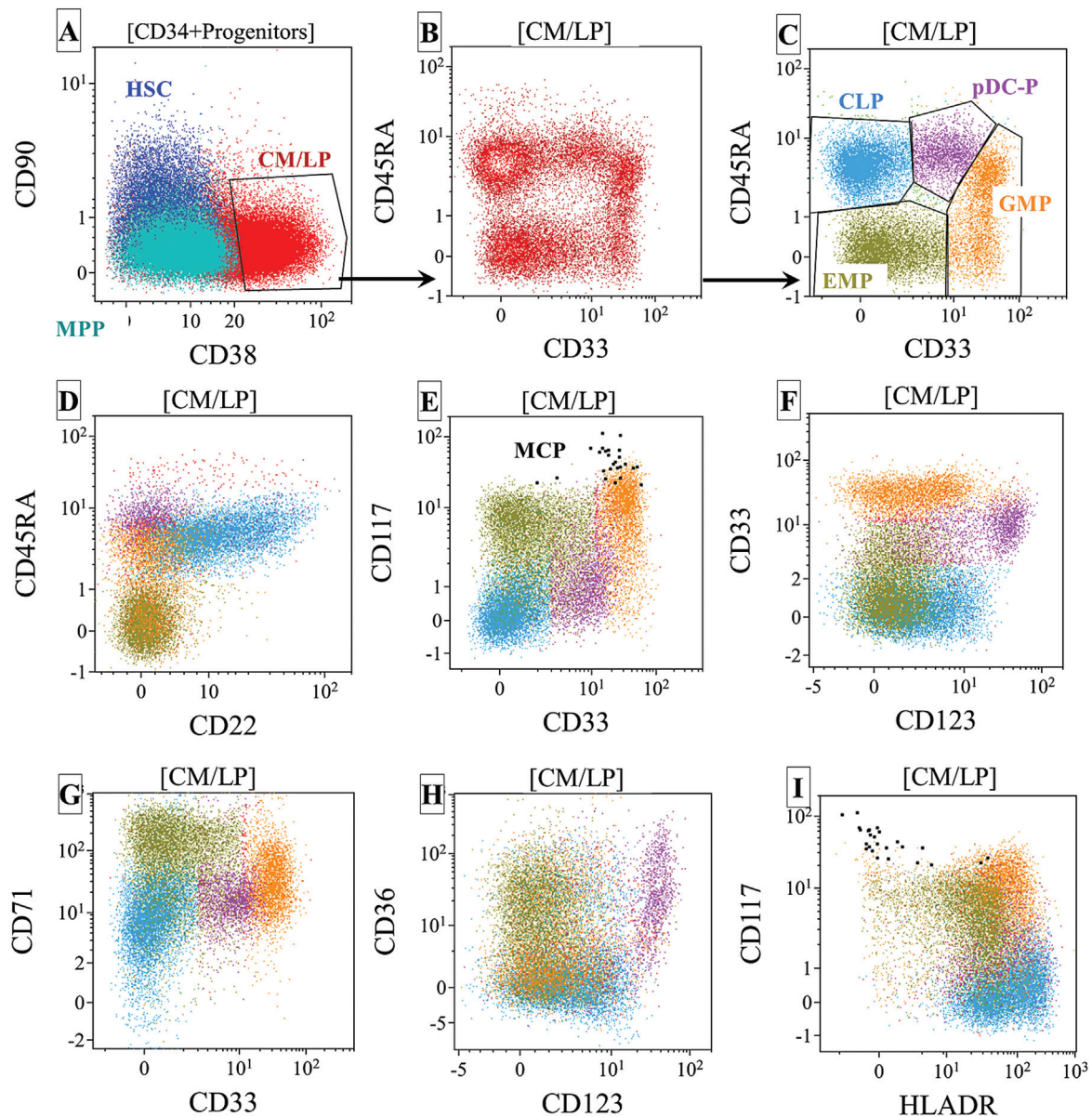
### Designing of Antibody Panel

#### Selection of Markers

AML MRD assessment is a relatively complex and challenging assay and primarily based on two universal approaches, that is, identification of leukemia-associated immunophenotype (LAIP) and DfN antigen expression.<sup>25,29,30</sup> Unlike B-ALL MRD where residual leukemic cells primarily needs to be distinguished from normal B-cell precursors, residual disease in AML needs to be distinguished from a group of common myeloid progenitor cells differentiating toward granulocytic, monocytic, erythroid, dendritic cell, basophil, and mast cell precursor cells.<sup>18,20,43</sup> Hence, in addition to backbone markers as recommended by the European LeukemiaNet (ELN) working group for AML MRD (such as CD45, CD34, CD38, CD117, and HLADR), the panel needs markers that can identify myeloid progenitor cells differentiating toward different hematopoietic cells.<sup>20,25,29,38</sup> Thus, antibody panel for AML MRD should also include CD13, CD15, and CD33 for granulocytic precursors; CD64, CD33, CD14, and CD36 for monocytic precursors; CD36 and CD71 for erythroid precursors; and HLADR, CD123, and CD203c for plasmacytoid dendritic cell, basophil, and mast cell precursors (→ **Supp. Fig. S1** and → **Supp. Table 1**, available in the online vesion). → **Figs. 1** and → **2** show the normal maturation stages of hematopoietic progenitors and their differentiation toward various sublineages with antigen expression patterns. The abnormal changes in the antigen intensities and asynchronous relation of these markers allow identification of aberrancies based on the DfN approach.<sup>27,35,44</sup> It should also include lymphoid markers commonly expression on leukemic cells for the detection of LAIP such as CD7, CD19, CD56, etc. Other rarely expressed lymphoid markers can also be included in the panel to further increase the applicability, such as CD2, CD4, CD5, CD11b, CD25, etc. A combination of CD34 and CD38 (CD34+ and CD38 +/-) also allows for evaluation of the earliest hematopoietic precursors, commonly referred to as hematopoietic stem cells. Overexpression of antigens such as CD33 and CD123 and aberrant expression of CD7 and CD56 can help identify abnormal hematopoietic stem cells, also referred to as LSC.<sup>45–49</sup> Additional markers can be added to identify more aberrancies on committed progenitor cells and stem cells, including CD45RA, CD52, CD54, CD96, CD97, CD366 (Tim-3), CD371 (CLL-1), etc.<sup>45–49</sup>

#### Antibody Panel Designing

For any flow cytometry assay, panel designing is mainly based on the configuration of flow cytometer, allowing a maximum number of colors for simultaneous analysis. Recently the ELN working group for AML MRD has recommended a minimum eight-color flow cytometry requirement for AML MRD assessment.<sup>29</sup> However, the utilization of flow cytometers with more colors such as 10, 12, 13, or 16 colors, and so on, can improve the assay further. Flow cytometers with more colors allow simultaneous evaluation of more markers; hence, the detection of more abnormalities that can increase the assay's



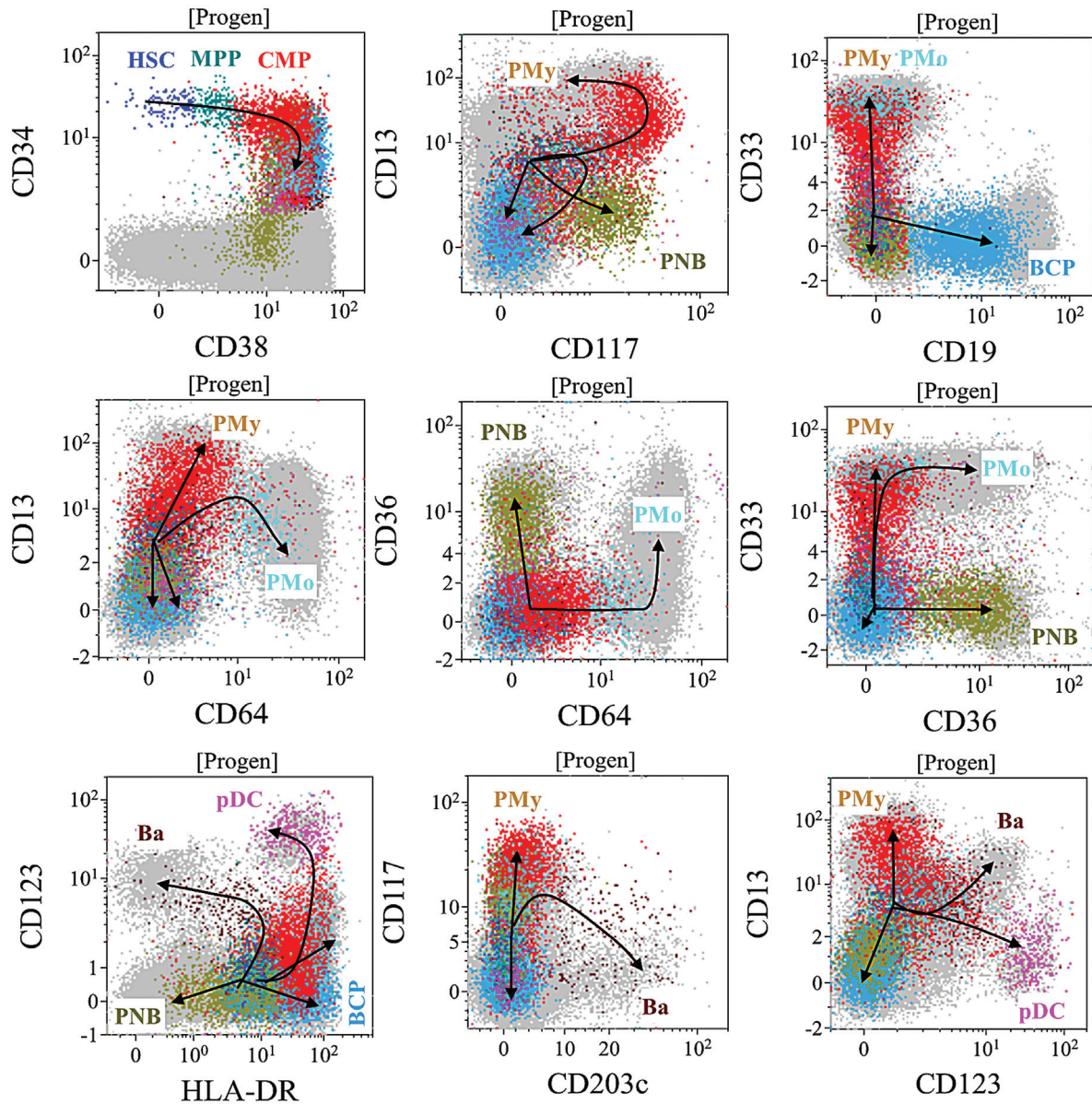
**Fig. 1** (A–I) Normal maturation stages of CD34+ hematopoietic progenitors and their differentiation toward various sublineages with antigen expression patterns. CLP, common myeloid progenitors; CM/LP, common myeloid and lymphoid progenitors; CMP, common myeloid progenitors; GMP, granulocyte–macrophage (monocyte) progenitors; HSC, hematopoietic stem cells; MCP, Mast cell precursors; MPP, multipotent progenitors; MEP, megakaryocyte erythrocyte progenitors; pDC-P, plasmacytoid dendritic cell precursors.

accuracy. The antibody panel of AML MRD requires a backbone of at least five common markers, that is, CD45, CD34, CD38, CD117, and HLADR.<sup>38</sup> A combination of these markers allows identification of progenitor cells and tracking of progenitor cells of interest in each tube. Other markers are required to identify progenitors differentiating toward various myeloid sublineages, identify deviation of antigen expression from normal, and detect LAIPs.<sup>38</sup> A common approach is to include those markers together, which can provide a sequence of maturation patterns of myeloid progenitor cells into sublineages. ▶ **Table 1** has shown a representative antibody combination that may be useful for AML MRD assessment on flow cytometers with various configurations. A recently pub-

lished multicenter study suggested that adding a customized antibody combination based on the diagnostic immunophenotype can improve MRD results further.

#### Sample Preparation, Processing, and Acquisition

The ELN working group for AML MRD has also provided recommendations on the technical aspects of AML MRD in detail.<sup>50</sup> Briefly, BM aspirate samples are ideal for AML MRD assessment although studies evaluating MRD in peripheral blood have been published. Peripheral blood usually has one log lower MRD levels than BM samples and hence, there is a risk of false-negative results in peripheral blood MRD assessment. A common concern in quality BM sample for MRD



**Fig. 2** Normal maturation patterns of CD34+ hematopoietic progenitors with their differentiation toward various sublineages using commonly used antigen expression. Each dot plot shows the differentiation of hematopoietic stem cells (dark blue dots) toward the respective committed precursors using a combination of different markers. The maturation sequences are shown with black arrows. Red dots indicate granulocytic differentiation, cyan blue dots indicate monocytic differentiation, olive green dots indicate erythroid differentiation, light blue dots indicate B-cell precursors, pink dots indicate plasmacytoid dendritic cell precursors, and brown dots indicate basophil precursors. The gray color dots indicate the background mature mononuclear cells from a commonly used CD45 versus SSC progenitor gate (shown in ►Fig. 1). Abbreviations: Ba, basophils; BCP, B-cell precursors; CMP, common myeloid progenitors; HSC, hematopoietic stem cells; MPP, multipotent progenitors; PMY, precursor myeloid cells; PMo, precursor monocytes; PNB, precursor normoblasts; pDC, plasmacytoid dendritic cell.

reporting is hemodilution, which also may lead to false-negative results. Hence, the first-pull (0.5–1 mL) BM aspirate is highly recommended for MRD monitoring. Samples should be collected in ethylenediaminetetraacetic acid (EDTA) or heparin anticoagulants, transported at 8 to 20° C and processed within 48 to 72 hours of collection. A bulk-lysis-stain method as described by the EuroFlow Consortium is a preferred method of processing for MRD assay although the stain-lyse-wash method is also used in a few centers.<sup>38,51</sup> An acquisition of a minimum of 500,000 CD45+ events per tube has been recommended; however,

the target of sample acquisition should be collection of the highest possible number of relevant events. It is always encouraged to acquire at least 1 to 2 million cells to increase the sensitivity of MRD assay and a minimum of 100 events of abnormal blasts to provide confidence in detecting MRD.<sup>18,29,30,35</sup>

#### Approach to MRD Analysis

An MFC-MRD analysis in AML is based on the integration of two approaches: (1) the LAIP approach and (2) the DfN approach.<sup>29,30</sup> Commonly available LAIPs can be divided

**Table 1** Common immunophenotypic aberrancies noted in acute myeloid leukemia with minimal residual disease monitoring

CD45 vs. SSC progenitor compartment	Different from normal (DfN)	Leukemia associated immunophenotype (LAIP)	
		Myeloid lineage associated	Lymphoid lineage associated
CD34 ++	CD203c- and HLADR-	CD34 +++	CD7 ++ <sup>a</sup> CD13 ++ CD36-
	HLADR ++ and CD117-	CD34 +/-	CD7+ CD38- or HLADR-
	CD13 ++, CD117 +, and CD33-	CD15 +, CD64-, CD13-	CD56 ++ <sup>b</sup> and/or CD7+
	CD13- and CD33 ++ CD15+	CD15 +, CD64 +, CD13-	CD56+ CD38-
	CD13+ and CD33 ++ CD117-	CD123 ++ CD117 +++	CD19 +, CD13-/+ , CD56+
	CD13 ++ and CD15	SS HLADR-	CD22 + CD117+
	CD123 ++ CD38-	SS CD117-	CD2 ++ <sup>c</sup>
	SS CD38-	CD117- and CD56+ or CD7+	CD5 ++ <sup>c</sup>
	CD33 ++ and CD38-	CD11b+	
	HLADR ++ and CD38-		
	CD117-/+ HLADR-		
CD34-CD117 ++	CD33 +++	CD123 ++ CD33 +++	CD56 +++
	HLADR +++/CD33 +++	CD203c-HLADR-CD13 ++	CD7+
	CD64 ++, CD36+	CD13- and CD33 ++ CD15+	
	CD33 ++ CD36-/+ and CD123 ++		
CD34-CD117-	CD33 ++, CD36-/+ , CD123 ++, and CD14-	CD64 ++, CD36 +, and HLADR-CD14-	CD56 +++ and CD33 +/CD13 +/CD64+
	CD13-CD15-CD33 ++ and CD14-	HLADR +++/CD45+	CD7+ and CD33 +/CD13 +/CD64+
	CD64 ++, CD36+ and CD33-/+ and CD14-	CD33 ++ HLADR-CD64-	
	CD13-, CD64 +, CD36 ++, and HLADR +++	CD33 + HLADR- and CD36 ++ CD42b+	

Note: (-) negative, (-/+) dim positive to negative, (+) dim positive, (++) moderate positive, (+++) strong positive; SS, significant subset.

<sup>a</sup>CD7 can be expressed in normal hematopoietic progenitors committed toward erythroid cells, plasmacytoid dendritic cells, and a small proportion of myeloblasts. It is usually weak and heterogeneous. The proportion of CD7 expressing CD34+ progenitors is usually higher in the CD13- part than the CD13+ part. However, if the CD7 + CD13+ proportion of CD34+ progenitors is higher than the CD13- proportion, it is highly suggestive of an aberrant population.

<sup>b</sup>CD56 can be expressed in normal myeloid progenitors in a regenerating bone marrow sample. It is usually weak and heterogeneous and expressed in a small subset.

<sup>c</sup>CD2 and CD5 expression is uncommon in acute myeloid leukemia (AML) blasts. They also show expression in normal myeloid progenitors in a regenerating bone marrow sample. It is usually weak and heterogeneous and expressed in a small subset.

into three groups. Group 1 focuses on the aberrant expression of lymphoid markers (e.g., CD2, CD7, CD11b, CD19, CD56, etc.), group 2 on the absence or overexpression of myelomonocytic markers (e.g., CD13, CD15, CD33, CD36, CD64, and CD71), and group 3 on aberrancies that include over-/underexpression of markers such as CD34, CD38, CD117, CD123, HLADR, etc.<sup>20,38,52</sup> These aberrancies are identified in diagnostic samples; hence, it is easy to follow in the MRD assessment. Sometimes, the residual leukemic blasts may show deviation in LAIP compared to diagnostic samples due to an immunophenotypic shift during therapy or predominantly show the immunophenotype expressed on a small subset of blasts at diagnosis due to heterogeneity in antigen expression.<sup>38,53-56</sup> Hence, evaluating multiple LAIPs at diagnosis is recommended to avoid false-negative results

due to the shift or absence of one LAIP. Consideration of at least two LAIPs to confirm MRD has been recommended.<sup>30,38,57</sup> A limitation to MRD assessment based on the LAIP-based approach is the need for a diagnostic immunophenotype, which may not be available sometimes, especially in stand-alone laboratories that receive referred samples. CD34+ progenitors from regenerating BM usually show weak expression of CD7 and sometimes CD56 and can lead to false-positive results.<sup>4</sup> Further, in a few cases, the LAIP approach may cause false-negative results if a new leukemic clone emerges during therapy or a chemoresistant subclone (less evident at diagnosis) persists. The DfN approach is very useful for MRD detection in such a scenario.

The DfN approach focuses on the difference in sequential expression of various antigens from normal progenitor cells,

their relationship with each other, that is, synchronous expression and abnormal changes in the intensities of antigen expression (underexpression or overexpression) compared to normal progenitors.<sup>30,37,38,57,58</sup> The DfN approach requires (1) an antibody panel with appropriate combinations of markers, (2) well-standardized and reproducible processing protocol and instrument setup, (3) an appropriately designed and updated MRD analysis template, and (4) adequate training and experience with detailed knowledge of immunophenotypic patterns of normal and regenerating BM progenitor cells. Thus, the approach to MFC-MRD depends on knowledge of normal patterns/sequences of various antigens and normal levels of their expression. This approach does not require knowledge of the immunophenotype of leukemic blasts at diagnosis and hence, the stability of the diagnostic LAIP during therapy may not affect the MRD results. **►Fig. 2** demonstrates the normal antigen expression pattern of antigen expression of commonly used markers in the AML MRD panel. A cluster of events (cells) showing deviation or difference from the normal antigen expression patterns can be considered abnormal blasts and part of MRD.<sup>57–60</sup> Usually, the immunophenotype of these cells coincides with the diagnostic immunophenotype. Recently, a few publications claimed the benefits of isolated evaluation of MFC-MRD based on immunophenotypic aberrancies in CD38-negative CD34-positive stem-cell-like compartment, also referred to as “leukemic stem cell–based MRD (LSC-MRD).”<sup>45,49,61,62</sup> It is merely an extension of MFC-MRD with a specific focus on CD38-negative CD34-positive progenitors and a study of a few additional markers (e.g., CD45RA, CD366, CD371, etc.) to identify more aberrancies. The data supporting its added value in predicting survival outcomes are limited and future studies are needed to provide robust data to incorporate in clinical practice.

### Gating Strategy

The initial part of the gating strategy for AML MRD follows a similar approach to that for immunophenotypic analysis for leukemia diagnosis. It starts with doublet discrimination using a scatter plot of FSC-H versus FSC-A, followed by exclusion of debris/checking for viability using FSC/SSC scatter plot, and defining the major populations based on the CD45 expression and SSC.<sup>38,59,63,64</sup> The leukemic blasts and progenitor cells can usually be identified using the weak CD45 expression and low SSC, expression of markers of immaturity such as CD34 and CD117, and absence markers of maturation, for example, CD11b, CD14, strong CD15 expression, etc. Other lineage markers can identify different subpopulations within myeloid progenitors, such as CD13 and CD33 identify granulocytic progenitors; CD33, CD64, and CD36 identify monocytic progenitors; CD36 in the absence of myeloid and monocytic markers identifies erythroid progenitors; CD123 and HLADR identify plasmacytoid dendritic and basophil progenitors, etc. The residual disease can be detected within these populations using a combination of markers based on the LAIPs (e.g., CD7, CD11b, CD19, and CD56) and/or DfN approaches (over-/underexpression of

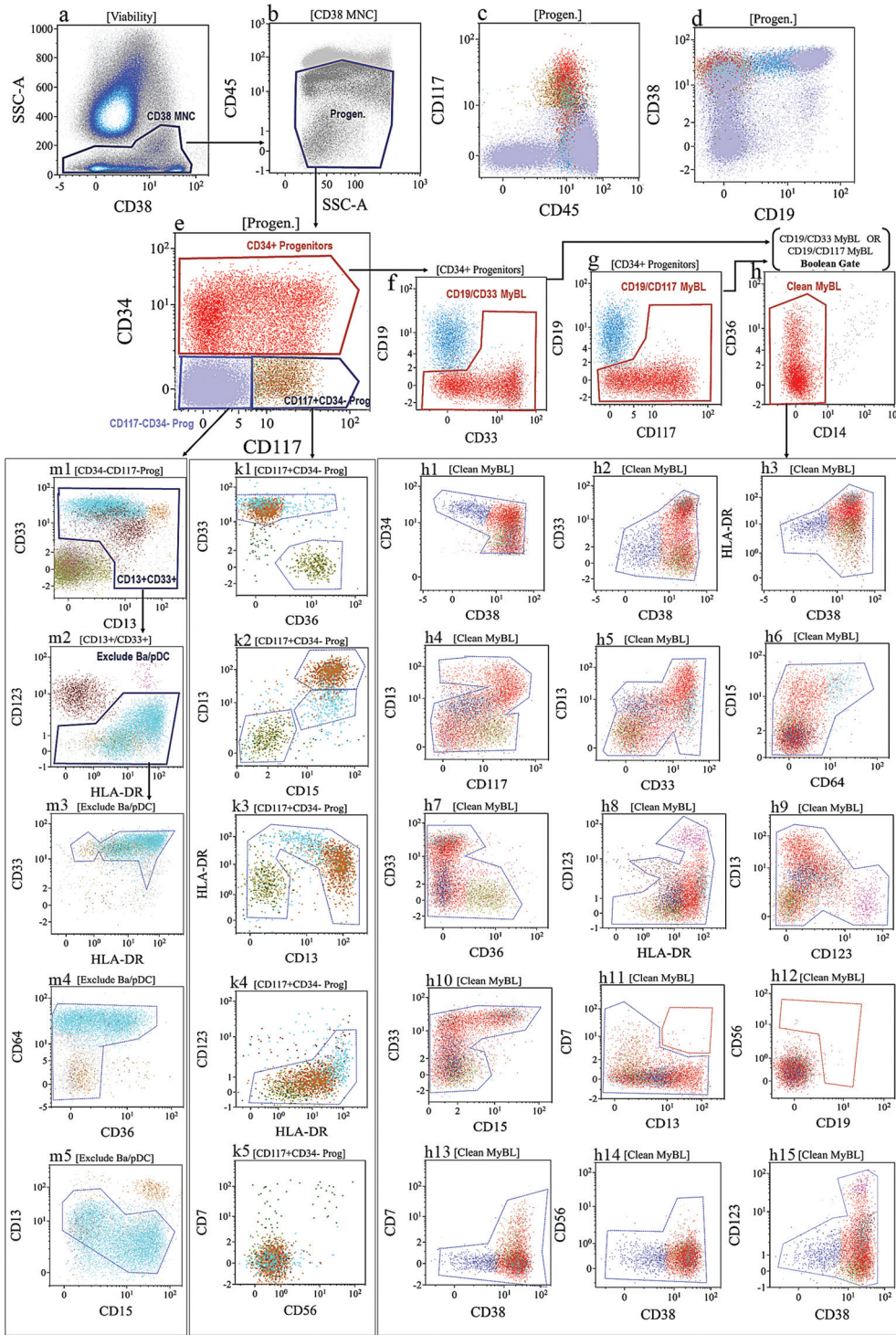
myelomonocytic markers, e.g., CD13, CD15, CD33, CD34, CD36, CD38, CD64, CD117, CD123, and HLADR).<sup>54,58</sup>

AML is an immunophenotypically heterogeneous and complex disease. Hence, to simplify the approach to MRD assessment, we have modified the gating strategy for AML MRD assessment in the Hematopathology Laboratory, Tata Memorial Centre (unpublished data). According to our strategy (shown in **►Fig. 3**), mononucleated cells (MNC) are gated after the exclusion of granulocytes with high side scatter using a combination of CD38 and SSC. After the exclusion of CD45 bright lymphocytes and monocytes, this MNC progenitor region is divided into three compartments utilizing a combination of CD34 and CD117: (1) MNC with CD34-positive expression, (2) MNC with CD117 positive but CD34 negative, and (3) MNC negative for both CD34 and CD117 expressions (**►Fig. 3**). This approach allows a focused assessment of progenitor cells within these three compartments. This approach emphasizes particular compartments based on the LAIP of CD34 and CD117. For example, if the leukemic blasts were CD34 negative and CD117 positive, then one can give more emphasis on that compartment. As shown in **►Supp. Table 2** (available in the online version), these three compartments contain fractions of subpopulations of progenitor cells differentiating to their mature forms. These populations and their maturation patterns are highlighted in **►Fig. 2**. Based on these normal maturation patterns, the progenitor cells from these three compartments are studied for DfN aberrancies and LAIPs including aberrant expression of lymphoid-associated markers such as CD7, CD19, and CD56 expression (**►Fig. 4**). **►Fig. 4** demonstrates examples of some AML MRD detected in CD34+, CD117+CD34-, and CD117-CD34-compartments from different patients. It has to be noted that a small subset of normal myeloid progenitor cells, especially in regenerating BM, usually show weak and heterogeneous expression of lymphoid markers such as CD2, CD4, CD7, and CD56.

Similarly, myeloid markers, such as CD13 and CD33, may show clustering in their expression in regenerating BM samples. Hence, it is essential to give enough consideration to this finding before labeling it an abnormal expression or LAIP. **►Table 1** highlights common immunophenotypic aberrancies and LAIPs, which have been useful in MRD detection in our experience (unpublished data).

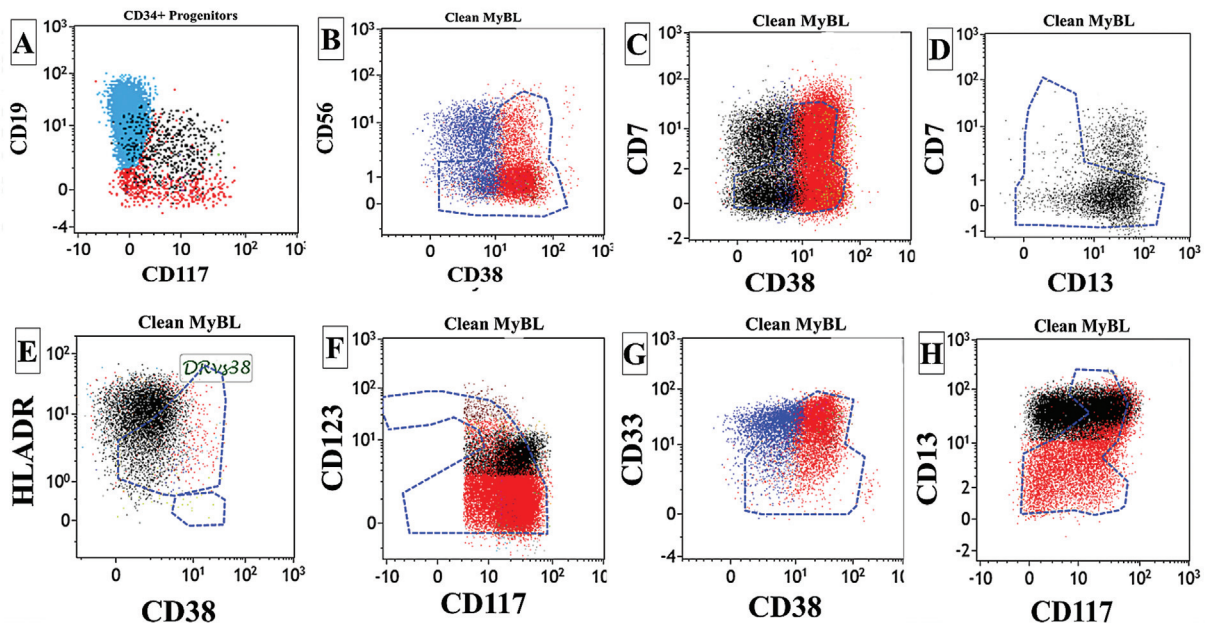
### Other Approaches

Recent few studies have suggested a database-guided MFC-MRD method to detect and characterize leukemic cells using Infinicyt software.<sup>65,66</sup> It uses an integrated LAIP and DfN approach to identify residual disease by comparing the given sample with six databases using automated tools, for example, principal component analysis. They have also listed the frequencies of various aberrancies found during this study. A major advantage of this approach is that it is less observer dependent and highly reproducible; however, a main disadvantage is that it requires a fixed antibody panel for MRD that has been used to create a database and Infinicyt software. A slight variation in instrument setup or processing may also

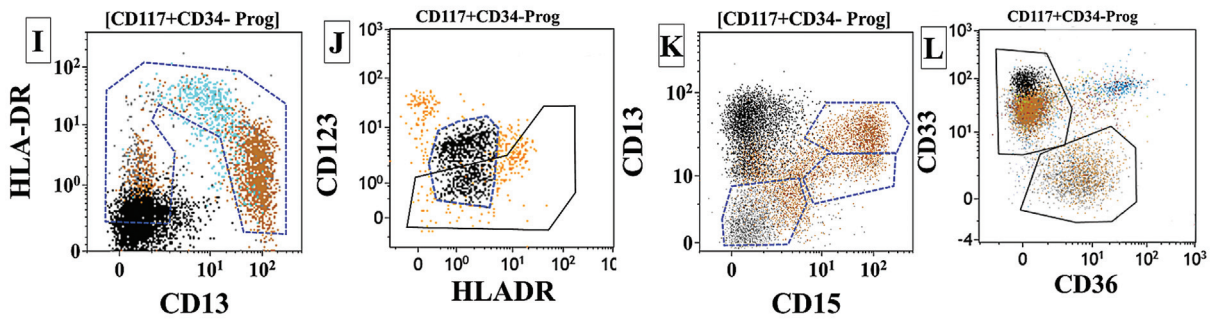


**Fig. 3** The gating strategy for acute myeloid leukemia (AML) minimal residual disease (MRD) used in Tata Memorial Centre, Mumbai. The mononucleated cells (MNC) were gated after the exclusion of granulocytes with high side scatter using a combination of CD38 versus SSC (dot plot [a]). After the exclusion of CD45 bright lymphocytes and monocytes, the MNC progenitor cells were gated using a “Progen” gate (dot plot [b]). Cells from “Progen” gate were divided into three compartments utilizing a combination of CD34 and CD117: (1) CD34+ progenitors, (2) CD117+ but CD34–progenitors, and (3) CD34–and CD117–progenitors (dot plot [e]). Normal B-cell precursors are excluded from CD34+ progenitors using a Boolean gate using a combination of CD19/CD33 MyBL (dot plot [f]) “OR” CD19/CD117 MyBL (dot plot [g]). These nonlymphoid CD34+ progenitors were further cleaned (Clean MyBL gate) after removing a few CD14 + CD36+ monocytes with nonspecific binding (dot plot [h]). These progenitors gated with “Clean MyBL gate” were studied for asynchronous and aberrant maturation patterns using a combination of various markers (shown in dot plots [h1] to [h15]) based on their normal maturation patterns (shown in ► Fig. 2). Dot plots [k1] to [k5] show the distribution of CD117 +/CD34–progenitors and their patterns to study asynchronous and aberrant maturation patterns. Dot plots [m3] to [m5] showed the distribution of CD117–/CD34–progenitors after excluding CD13/CD33 negative (dot plot [m1]) and basophils (brown dots)/plasmacytoid dendritic cells (pink dots; dot plot [m2]). Note: ► Fig. 4 is a representative example of the AML MRD approach used by the author. Demonstration of a complete approach is not possible due to limited space for figures.

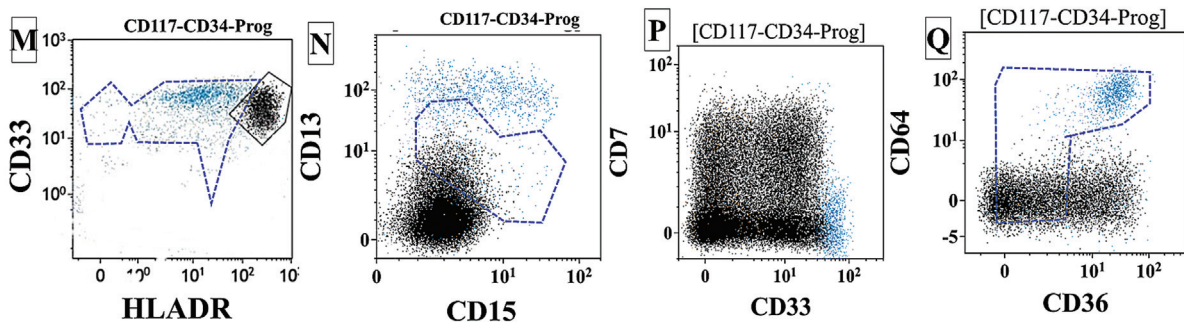
**Examples of MRD in CD34+ Progenitor compartment**



**Examples of MRD in CD117+CD34- Progenitor compartment**



**Examples of MRD in CD117-CD34- Progenitor compartment**



**Fig. 4** Dot plots (A–H) show some examples of AML MRD detected in the CD34+ progenitor compartment, (I–L) in CD117+CD34- progenitor compartment and (M–Q) in CD117-CD34- compartment from different patients. AML MRD is indicated with *black dots* in all dot plots except in dot plots (B) and (G). Here, MRD is indicated with *dark blue dots* showing aberrant CD56 expression (B) and CD33 overexpression (G) in CD38-negative stem cells.

lead to erroneous results. Another multicenter study by Röhnert et al suggested an alternative method based on the levels of immunophenotypic subpopulations within progenitor cells exceeding the reference values established using leukemia-free controls.<sup>50</sup> They identified 32 immuno-

phenotypic subpopulations with aberrant phenotypes using an eight-color panel utilizing a hierarchical gating strategy with fixed gates to develop a clear-cut LAIP-based DfN approach. Although this method appears simple and reproducible, it is challenging to establish reference values of



various immunophenotypic subpopulations with a comprehensive antibody panel, especially in posttherapy regenerating BM samples at different time points and for different age groups. Combining these new approaches to the template-based analysis within a particular setup may improve the MFC-MRD further.

### Quality Control

The laboratory must follow the standard protocol for daily calibration and quality control for flow cytometry instruments. Instead of using an individual antibody, it is highly recommended to use a prevalidated antibody cocktail to avoid pipetting errors and to provide consistent results.<sup>38,58,63</sup> An MRD template should be designed with at least 20 control BM samples (10 nonleukemic and 10 regenerating samples). Since the instrument settings may show some variation after a while, running control samples regularly (at least once in a month) and updating the template is highly recommended.<sup>58,67</sup> Participating in workshops and Continuous Medical Education programs (CMEs) and an external quality assurance (EQA) program whenever available is also advised.

### Limit of Detection/Lower Limit of Quantitation (LLOQ)

Since AML is an immunophenotypically heterogeneous disease, it is practically challenging to establish and apply limit of detection (LOD) and lower limit of quantitation (LLOQ) for MFC-MRD in routine clinical practice.<sup>68</sup> However, the ELN has recommended performing LOD and LLOQ for each antibody panel combination whenever possible, especially if required by regulatory authorities for making clinical decisions.<sup>29,38</sup> Unlike acute lymphoblastic leukemia and multiple myeloma, AML residual leukemic cells can have significant immunophenotypic overlap with normal BM progenitor cells.<sup>63,64</sup> Such overlap depends on the degree of LAIPs separating tumor cells from normal BM progenitor cells. LOD and LLOQ established based on one particular immunophenotype may not apply to AML with different LAIPs. LOD and LLOQ in AML heavily depend on the antibody panel with markers and their respective combinations providing LAIPs and DfN immunophenotype. Hence, establishing immunophenotype-specific LOD/LLOQ is being considered, but it is complicated and challenging.<sup>68</sup> Future studies on such immunophenotype-specific LOD/LLOQ can highlight its practical utility. A technical guide by the ELN working group on AML MRD has recommended a cluster of 20 events with more than one LAIPs to define LOD and a cluster of 100 events for LLOQ for MFC-MRD assay.

### Clinically Significant Level of MRD and Time Points

The ELN working group on AML MRD has recommended a cutoff of  $\geq 0.1\%$  of CD45-expressing cells as MFC-MRD positivity threshold for clinical decision-making.<sup>25,29</sup> However, several studies have suggested MRD levels below 0.1% can also reliably predict DFS and a few studies have also shown the predictive value of “any detectable level” for MRD positivity in AML.<sup>2,11,16,19,20,24,26,29,33,42,44,50,52,57,60,66,68</sup>

The usual recommended MFC MRD assessment time points for BM samples are after two cycles of chemotherapy (postinduction), at the end of consolidation (postconsolidation), and before and at day 100 post-stem cell transplantation, if applicable.<sup>24,25,27–29,31,34,38,42,57,69–83</sup>

### New Advances in MFC-MRD

Currently, available data for MFC-MRD in AML are predominantly limited to 8- to 10-color flow cytometry assay that restricts the utility of combinations of different markers to existing 8- to 10-color antibody combinations limiting the number of LAIP or DfN approaches to few combinations. Flow cytometers with the capability of more colors (13 or 16 or more colors) allow the utilization of more markers simultaneously and thus can improve the accuracy of the MRD assay. The availability of new technologies such as CyTOF mass spectrometry and full-spectrum flow cytometry (FSFC) has demonstrated the capacity to utilize many markers (up to 27 colors) for AML MRD.<sup>84,85</sup> Additionally, by increasing the number of events from 1 million to 10 million, the sensitivity of MRD assay can easily be reached to as high as  $2 \times 10^{-6}$  (0.0002%) MRD detection.<sup>63</sup> Thus, the recent availability of new flow cytometry technologies and newer dyes has improved the assay sensitivity and specificity for MRD assessment by flow cytometry.

### Conclusion

The clinical value of MRD assessment in AML has been established beyond doubt. The ELN working group for AML MRD has given a list of recommendations on the technical aspects and its incorporation in clinical trials and routine practice. AML MRD still has uncertainties regarding optimal antibody combinations, timing and frequency of measurements, thresholds for MRD positivity, and techniques. Further, there are challenges like technical optimization and expertise in interpreting MRD and training resources, etc. Despite these uncertainties and challenges, studies have consistently shown that the presence of MRD after induction or consolidation therapy or around the time of hematopoietic stem cell transplantation identifies patients at high risk of disease recurrence and short survival, even after adjusting for other risk factors. AML resistance and relapse result from a complex interplay between leukemic and immune cells, which is not considered by current MRD measurements. Dependable assessment of MRD may require an integrated approach combining MFC-MRD and molecular techniques. Thus, MRD testing needs more standardization and validation utilizing advanced technologies and incorporating additional factors such as immune cells and a multiple-technique approach before being used as surrogate endpoints in clinical trials and real-life clinical practice.

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### Conflict of Interest

None declared.

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