Voyage of Measurable Residual Disease Assessment in Multiple Myeloma Using Multiparametric Flow Cytometry

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Abstract

Measurable residual disease (MRD) in multiple myeloma (MM) has emerged as one of the strongest and independent biomarkers to evaluate therapeutic response for the prediction of long-term treatment outcome. With the incorporation of MRD in response assessment criterion by International Myeloma Working Group, it has become the routine parameter to be assessed at various time points after therapy. Among various techniques to assess MRD, multiparametric flow cytometry (MFC)-based MRD estimation has evolved dramatically over the last two decades achieving sensitivity comparable to molecular methods. Next-generation flow cytometry with the incorporation of innovative tools in MRD detection including consortium-based guidelines for preanalytical and analytical factors led to the overall improvement in MFC-based MRD detection. However, flow cytometry assays suffer from inherent challenges ranging from procedural hemodilution to lack of harmonization and standardization across the centers.

This review article outlines and summarizes the essential laboratory prerequisites for reproducible MRD analysis by flow cytometry. Furthermore, a brief account of the utility of MRD evaluation in clinical practice as predictor of response and long-term treatment outcome has also been discussed. Considering the evolution of MFC-based MRD over two decades from a scientific research tool to a routine clinical diagnostic assay, it needs to be explored further in studying complex phenomenon like clonal evolution, clonal switches, and identification of treatment refractory clones for guiding more effective therapies improving overall survival.

Keywords

- multiple myeloma
- flow cytometry
- next-generation flow
- measurable residual disease

Introduction

Multiple myeloma (MM) is a hematological malignancy characterized by the proliferation of clonal plasma cells (PC) in the bone marrow (BM) and presence of monoclonal protein (M protein) in serum and/or urine and lytic bone lesions. Though the development of novel therapeutic strategies has improved the overall prognosis, a substantial number of patients relapse despite achieving good clinical response. Following the current guidelines of response assessment in MM, over two-thirds of the patients achieving complete remission (CR) relapse within 2 years.[1,2] Thus, in order to refine the response assessment in MM, International Myeloma Working Group (IMWG) included the measurable residual disease (MRD) assessment as additional response criteria in 2016.[3] MRD in MM is defined as the residual small

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number of cancer cells surviving after treatment that is not identifiable by routine clinical and laboratory parameters.

IMWG recommends either intramedullary or extramedullary MRD detection based on BM aspirate (BMA) samples and imaging techniques respectively.[3] Extramedullary MRD detection includes imaging techniques, such as positron emission tomography with computed tomography using ^{18}F-deoxyglucose or magnetic resonance imaging.[4]

Earliest evidence of MRD assessment in MM came in late 1980s when an attempt was made to detect residual PC in patients' BM biopsies using immunohistochemistry, though the technique was too crude to estimate MRD.[5] Following this, researchers in the initial years estimated MRD in MM by qualitative polymerase chain reaction (PCR) followed by quantitative allele-specific oligonucleotide PCR (ASO qPCR).[6,7] Use of multiparametric flow cytometry (MFC) for MRD detection in MM was attempted in 1999, when Almeida et al explored the role of flow cytometric immunophenotyping (FCMI) and DNA ploidy status for the investigation of residual neoplastic PC in MM patients.[8] Since then, MFC has contributed significantly to the understanding of the aberrant/clonal PC (APC) compartment and has now found a place in the diagnosis, prognosis, and in treatment monitoring of MM. In this review, we will critically appraise the role of MFC for MRD detection in MM and make an attempt to identify the caveats present in MM MRD estimation by FCMI.

Flow Cytometry in Myeloma MRD—Transitioning from Conventional Flow Cytometry to Next-Generation Flow cytometry

MFC has become the most valuable tool to monitor MRD and evaluate the depth of response in MM patients. MFC offers an edge over other techniques of MRD detection, that is, ASO qPCR and next-generation sequencing (NGS) due to its rapid turn-around time, wider applicability, and cost-effectiveness. Furthermore, it plays a crucial role in treatment monitoring and response assessment in oligo-secretory and nonsecretory myeloma where monoclonal protein estimation has no role. FCMI has also shown a promising role in demonstrating rare phenomenon like clonal switch and light chain escape. The very first step in the FCMI for MRD evaluation is optimal panel design and validation before using it routinely in clinical diagnostics.[9] Four set of markers are required for a panel to precisely determine MRD:

A. Gating marker—CD38, CD138, and CD45
B. Markers of aberrancies—CD19, CD56, CD27, CD81, CD117, CD20, CD28, etc.
C. Markers to identify clonality—cyt kappa and cyt lambda
D. Markers for adequacy—CD117. The major limitation of four to six color panels is lack of incorporation of all sets of markers in a single tube that led to the transition to higher versions with added benefits of increased sensitivity.

Conventional 4–6 Color Panel

Though the conventional four to six-color flow-MRD is applicable in majority of patients (≦95%), the sensitivity of conventional flow cytometry remains lower ($<10^{-4}$) than that of molecular techniques, namely ASOqPCR ($<10^{-5}$) and NGS ($<10^{-6}$).[10–12] Due to the limited sensitivity of four to six color assays, there was high likelihood of missing the low APC burden. Limitations of such limited panel assay are high likelihood of missing the low APC burden and failure to combine immunophenotypic aberrancies with light chain restriction leading to difficulty in the characterization and differentiation of APC from non-neoplastic normal PC (NPC). Thus, overall, the four to six-color panel was not suitable for MRD assessment in MM and the transition to higher version was inevitable.

Conventional 8-Color Panel

A step forward in disease monitoring was conventional eight-color flow-MRD assays with an increased sensitivity of less than 10^{-4} to less than 10^{-5}, leading to a significantly improved prediction of outcome across different studies.[9,13,14]However, the two major limitations of the conventional eight-color antibody panel were limited sensitivity and lack of standardization in terms of variation in antibodies, number of cells evaluated, and lack of unanimous cutoff for MRD levels. To offset this challenge, standardization efforts were made by the Euroflow consortium for highly sensitive and standardized detection of MRD in MM using the NGF approach.[15]

Euroflow 2-Tube-8-Color Panel

Euroflow standardization study for NGF included an optimized 2-tube eight-color antibody panel, acquisition of more than or equal to10^7 cells/sample using bulk lysis procedure and construction of novel software tools for combined analysis of both the tubes for automated PC gating. In this multicenter evaluation of NGF for myeloma MRD, one-fourth (∼25%) patients classified as MRD-negative by conventional eight-color flow cytometry were found to be MRD-positive by NGF. This resulted in identification of a flow MRD-negative subset of patients within the CR group who had significantly superior progression-free survival (PFS). However, in the real-life clinical settings especially in the resource constraint countries, the inherent challenges associated with Euroflow two tube panel are as follows:

A. High sample demand with wastage: Two eight-color tubes with the acquisition of more than 1 million cells per tube require a higher volume of BMA that may not be always available due to the primary fibrotic nature of MM. Furthermore, it also leads to wastage of precious samples which can be utilized for cytogenetic and molecular studies.
B. Turnaround time: Comparatively longer due to increased sample processing and data acquisition time
C. Wastage of antibodies: Common gating markers in both the tubes
D. Increased technical demand: Heavy demand on sample quality control, and data storage
E. Analysis: Time-consuming with inferential reasoning between the tubes. As a result, investigators started looking for a better, cost-effective, and efficient panel for MRD evaluation in MM.

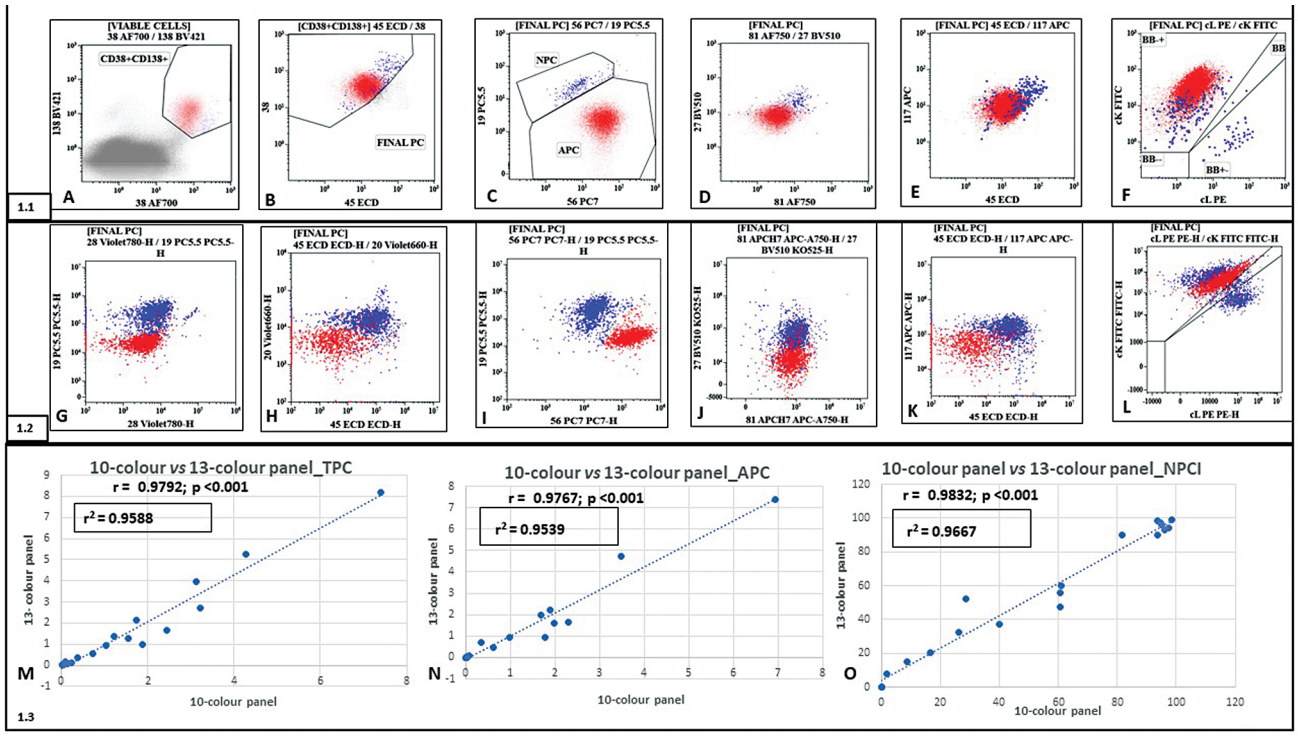

Fig. 1 Flow cytometric dot plots showing PC quantification using 10- and 13-color panel, respectively (1.1 and 1.2); Bland-Altman correlation analysis showing linear positive correlation between 10-color versus 13-color panel (1.3). APC, aberrant PC; NPC, normal PC; PC, plasma cell.

Single-Tube-10-Color Panel

In an attempt to develop less expensive, low sample requisite method to detect MRD with equivalent sensitivity to that of Euroflow-NGF, single-tube-10-color panel was designed across few centers.[16,17] In a study by Sato et al,[17] the analytical ability of the10-color panel was compared to that of Euroflow-NGF that demonstrated a good correlation between the two methods. This study also demonstrated a comparable performance of 10-color-MFC and Euroflow-NGF in terms of median percentages of total PC (0.2148 vs. 0.2200%, $r = 0.950$) as well neoplastic PC (0.0012 vs. 0.0007%, $r = 0.954$). Thus, as per the published literature, the single tube10-color-panel is a promising alternative to Euroflow two tube eight-color panels for MRD detection.

Beyond 10-Color Panel in MM

In constructing the panel for MRD detection, the next relevant question is the need of additional immunophenotypic markers for MRD evaluation. In a recent study, fair discrimination between normal and clonal PC was demonstrated in most of the plasma cell proliferative disorders (PCPD) samples (~99.5% of samples) using eight markers (Gating:CD38, CD138, CD45; aberrancy:CD19, CD56, CD27; clonality: Cy-kappa & Cy-lambda).[18] This confirmed the high efficacy of single tube-10-color-panel in APC identification and thus, MRD detection. Our in-house data for 13-color panel with addition of CD20, CD28 and CD3 (unpublished) showed a good correlation between single-tube-10-color panel (median TPC: 0.98%, range: 0.02–5.24; median APC: 0.59%, range: 0.009–4.72; median neoplastic plasma cell index [NPCI]: 54.25%, range: 8.16–99.11) and single-tube-13-color panel (median TPC: 1.12%, range: 0.03–4.28; median APC: 0.48%, range: 0.01–4.39; median NPCI: 60.55%, range: 1.65–98.46; ►**Fig. 1**) with no added benefits of more than 10 color panel in MRD analysis. Nonetheless, a higher version of panel can be explored for ancillary immunophenotypic studies, namely B-cell subset analysis; immune cell profiling in MM patients owing to the benefits of additional markers. ►**Supplementary Table 1** (available in the online version) shows various antibody panels used for flow cytometry based MRD assay in MM.

Preanalytical Considerations for MFC-Based MRD Detection

The basic step in the MRD monitoring of MM is to ensure optimal sample quality till the processing of sample (►**Supplementary Fig. 1**, available in the online version).

Sample Type and Collection

BMA sample is the prerequisite for MRD estimation; however, peripheral blood sample has been explored as an alternative for circulating PC identification and quantification using sequencing studies across the literature.[19–21] The major drawbacks associated with the use of bone marrow sample for MRD detection are use of quasi-invasive BM aspiration procedure, inadequate PC representation due to the inherent marrow fibrosis associated with MM, and procedural hemodilution. The first two issues can be addressed with the use of non-BMA-based alternate strategy to detect MRD, that is, estimation

of M-protein and imaging.[22,23] Hemodilution is one of the major challenges in MRD estimation and can be reduced using first pull BMA sample for MFC assay as shown across various studies.[24–26] For sample collection, ethylenediaminetetraacetic acid (EDTA) is the anticoagulant of choice. Although heparin can be used as an alternative to EDTA in MRD estimations in other hematolymphoid tumors, its use in MM is best avoided as it interferes with assessments of CD138 that is a key gating marker in myeloma MRD. The use of sodium citrate is also avoided as it reduces the cell viability.[27]

Sample Storage and Transportation

Apart from the type of anticoagulant used, the handling and transportation of the specimen are critical. The peripheral blood and BM specimen must be insulated from the external temperature. While transportation, the age of the specimen also plays an important role, therefore, the specimens must be labeled with the date and time of collection. A 24-hour cutoff is considered appropriate for sample processing to ensure PC viability for accurate MRD determination.[9] However, for all practical purposes a 4 to 6-hour cutoff from sample collection to sample processing is preferred. The viability of the specimen should be assessed with the flow cytometric evaluation and the samples with less than 85% viability should be reported stating that the viability of the sample was suboptimal for testing.[28] Factors indicating a compromised specimen are hemodilution, clots, small BMA volume, and cold or excessively warm samples.

Sample Processing

BMA sample for MRD detection should be preferably processed within 4 to 6 hours of collection to get maximum PC yield; however, it may be stored for 2 days at 4 to 8 degrees. Lysing the sample before staining is preferred especially in samples for MRD analysis as this allows the delivery of maximum number of cells per tube in the panel. However, this method also has a drawback that certain antibody-fluorochrome **c**onjugates become unstable. Cell fixation post-staining is recommended for resolving such issues.[29] The samples can be gently prelysed by incubating with ammonium chloride-based lysis solution. Another method is used is lysis of the sample post-staining, but it is associated with lower stability issues and decreased sensitivity due to lower number of cells. Euroflow consortium also supports the prelysis method for MRD detection with high cellular yield in MM and the same method can be used for PC enumeration in other PCPDs.[30]

Analytical Considerations for MFC-Based MRD Detection

Determinants of Reproducible MRD Enumeration

Multicenter data has demonstrated that the level of 0.01% is of clear prognostic value for MRD monitoring in MM.[31] With the advancement in the technology, the assay sensitivity has increased to 10^{-5} to 10^{-6} and with the improvements in therapy, the current minimum acceptable sensitivity is 0.001% and the recommended threshold for abnormal PC in the determination of MRD ranges from 20 to 100 cells.[32,33] A number of studies have demonstrated that a cluster of 20 cells is a conservative value for the smallest number of a homogenous and clustered population of cells that can be reliably detected by an experienced Hematopathologist.[9,15,17] The limit of blank (LOB), the limit of detection (LOD) and the lower limit of quantitation (LLOQ) are further determined to establish the sensitivity and reproducibility of the flow cytometric assay for MRD assessment.[9] LOB is calculated as LOB = mean of blank values plus 1.645 x (Standard deviation [SD] of blank values).[34] The LOD is calculated as LOB plus three times SD of blank values.[34] LOD determined using the values from the LOB experiment may further be validated with a dilution and spiking experiment. In the experiment for LLOQ determination, spiked and diluted cells with the same concentration (number) of neoplastic PC are processed and acquired multiple times separately to determine the minimum number of clonal PC present in the sample that can provide assay results with CV less than 20%.[16] Next critical determinant in MRD calculation is assigning appropriate numerator and denominator for MRD estimation. For all practical purposes, numerator is clonal PC showing light chain restriction with/without aberrant immunophenotype designated as APC in the subsequent sections. The denominator used for MRD studies is total viable cells that are total events after removal of doublets and debris and thus, MRD = clonal PC/ total viable cells. Another parameter that has been shown to be relevant in MRD estimation in MM by our group is total PC. The use of total PC as denominator is based on the premise that in a hemodiluted BM, both the normal and neoplastic PC are equally diluted and thus this statistic offsets the effect of hemodilution to some extent. Thus, using clonal PC as numerator and total PC as denominator, we defined NPCI, as NPCI = clonal PC/total PC.[35]

Data Acquisition and Analysis Strategy

A number of clinical studies have supported the fact that the total number of events acquired is a key step in the interpretation of the specimen quality for MRD negative cases with the acquisition of at least two million cellular events as the acceptable minimum number in the absence of MRD.[9] If MRD is not detectable and the total cellular events acquired are fewer than two million, both LOD and a qualifying statement as to the decreased level of sensitivity should be mentioned in the final MRD report.[28] Data acquisition is considered to be accomplished when the acceptable total cell collection is achieved, and the specimen meets the criteria of quality. For samples in which MRD is not detected, the quality of the specimen must be checked by an assessment of normal cell population such as mast cells, B cell progenitors, NPC, nucleated red blood cells, and/or myeloblasts (►**Fig. 2**).[26,36,37] After ensuring BMA adequacy and acquiring enough events, the next critical step is the gating strategy for identification of the PC compartment. The backbone gating markers for the PC analysis strategy are CD38, CD138, and CD45.[38,39] However, in the era of targeted therapy especially in the setting of use of anti-CD38 monoclonal antibody in the

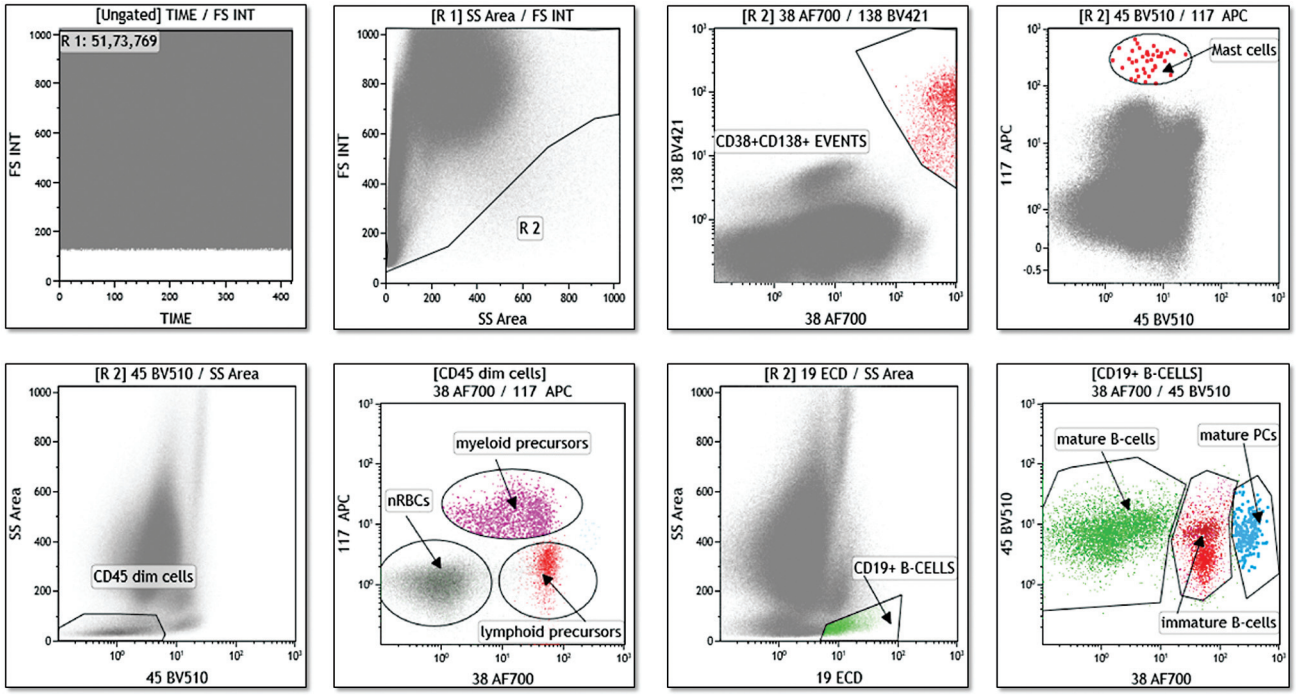

Fig. 2 Flow cytometric dot plots showing various components used to measure adequacy of BM in MRD assessment. BM, bone marrow; MRD, measurable residual disease.

treatment protocols, a search for alternate gating markers for PC continues. In quest of newer markers for PC identification, Pojero et al carried out a detailed analysis of several PC-associated markers and demonstrated that CD54 and CD319 had limited utility for PC identification because of significant overlap of the staining on PCs and other myeloid cells in the sample.[40] Furthermore, CD229 was found to be reliable marker for the identification of PCs in patients undergoing anti-CD38 or anti-CD138 therapy. Recently, Broijl et al explored the role of CD38-multiepitope antibody and the VS38c antibody for MRD assessment in MM patients and concluded that both antibodies allow reliable MRD detection with 100% concordance with added benefits of VS38c in daratumumab-treated patients due to its intracellular expression.[41] ►**Fig. 3** demonstrates the gating strategy for PC identification using four gating markers and shows that best discrimination between PC and non-PC compartment is achieved with the combination of CD38 and CD138. The utility of CD229 as a gating marker for PC identification reaffirms its role in MRD detection in patients receiving anti-CD38 therapy.[42] Overall, the combination of CD38, CD138, and CD45 remains the mainstay for PC gating and additional markers may help in specific circumstances.[39]

Characterization and Discrimination of Normal from Aberrant PC

After ensuring the optimal gating strategy, the next critical step in MRD evaluation by MFC is the discrimination of nonmalignant polyclonal NPC from the malignant APC. There is no single surface marker that would enable 100% discrimination of benign PCs from myeloma PCs, highlighting the need of multiple markers to differentiate NPC from APC. A major challenge in the immunophenotypic characterization of APC is the presence of a polyclonal reactive PC that closely mimics the immunophenotype of neoplastic PC. With the progressive increase in the number of markers and events analyzed, we are increasingly becoming aware of the existence of polyclonal PCs with immunophenotypic aberrancies and therefore, an in-depth understanding of the immunophenotypic heterogeneity of the background NPC is essential. Recent studies have shown evidence of wider immunophenotypic variation in NPC than earlier studied, thus, revealing a partial overlap between the NPC and APC.[43,44] Immunophenotypic aberrancies in polyclonal PCs have demonstrated for all the markers using 10-color panel, signifying that an aberrancy of immunophenotypic marker alone does not define malignancy in otherwise polyclonal population of PCs.[16] Thus, in view of the high frequency of immunophenotypic aberrations observed in polyclonal PCs, presence of aberrant immunophenotype alone can no longer be used to define clonal APC and, thus antigen aberration must be supported by light chain restriction for correct assessment of MRD.

Impact of Therapy on MRD Assessment in MM

Data on immunomodulation of the PCs subsequent to antimyeloma therapy is sparse.[45,46] However, it may be noted that the discriminative power of an individual antigen for delineating neoplastic from non-neoplastic PC varies from case to case and is not stable during the disease course of treatment. Almost a decade ago, we reported immunophenotypic changes in clonal APC following antimyeloma therapy in as many as78% cases.[47] In this context, it is important to note that the high degree of immunomodulation renders the baseline diagnostic immunophenotype redundant for the

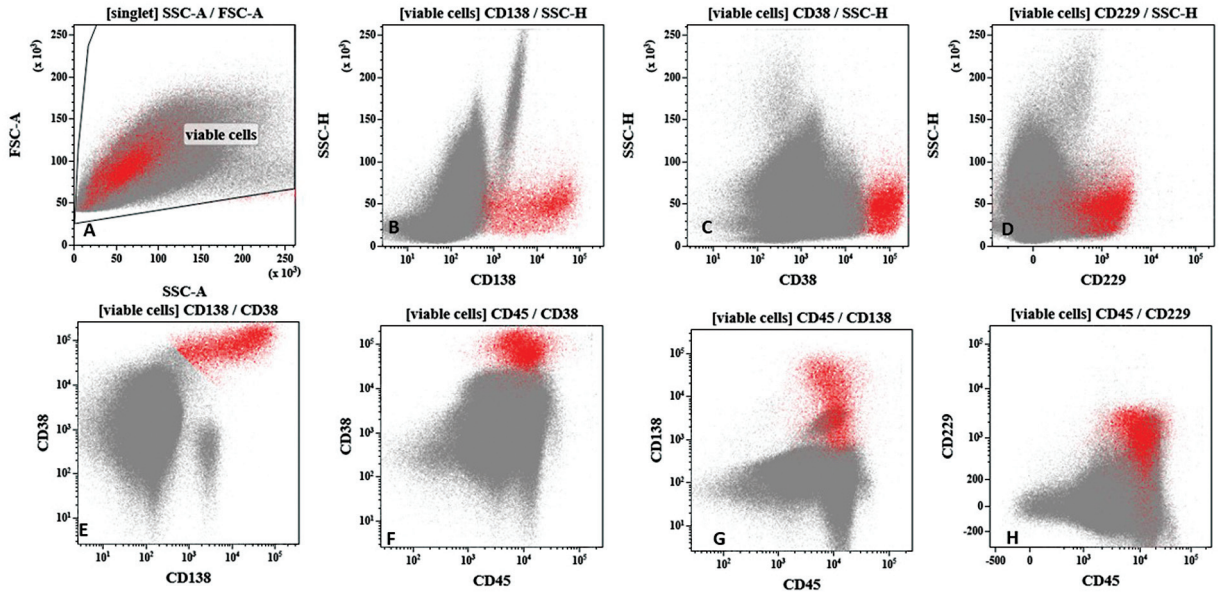

Fig. 3 Flow cytometric dot plots showing gating strategy; maximum PC yield with CD38 versus CD138 (E) followed by CD38 versus CD45 (F); CD229 show overlap with non-PC compartment (D) and (H) reconfirming CD38 and CD138 as PC specific marker. PC, plasma cell.

purpose of MRD assessments in MM. CD45, CD38, and CD138 are gating markers and thus change in their expression is likely to affect the MRD as recently described.[16] Thus, more prospective studies in a larger cohort of patients may unravel the mechanism of post-therapy immunomodulation under the aegis of various treatment modalities and lines of therapy administered.

Caveats and Pitfalls of Flow-Based MRD Assessment in MM

Since MRD emerged as one of the strongest and independent prognostic indicators relating to long-term treatment outcomes across various studies, accurate and precise determination of MRD is crucial for disease monitoring.[48] There are multiple factors influencing the MRD status in MM patients on therapy.

Hemodiluted Bone Marrow Aspirate

This is the major pitfall in assessing MRD across laboratories as it limits the minimum number of neoplastic cells required to detect MRD. To increase the challenge, high throughput techniques like NGF require high amounts of starting material. Several measures have been taken in the past to overcome this challenge including use of first-pass sample for MRD assessment, bulk lysis as the optimal preanalytical procedure, and evaluating sample cellularity (e.g., by quantification of erythroblasts more than 5% and mast cells by flow cytometry or smear) before proceeding with MRD evaluation. However, none of these methods found to be beneficial in all cases. Thus, recently we demonstrated the utility of NPCI, that is, the percentage of clonal APC of the total PC in for MRD evaluation as a parameter to document MRD.[35] Since both neoplastic and NPC are proportionately reduced in a hemodiluted BMA, NPCI may prove to be a better parameter to assess MRD and may be explored further in larger cohorts.

Multiple Institutional and Nonstandardized Protocols

This is one of the major pitfalls in the MRD detection in MM. A survey of multi-institutional practices for MFC based MRD detection in MM revealed that MRD estimation suffers from major heterogeneity with considerable variation in the number of BM cells analyzed (events) and the number of APC needed to identify the MRD.[49] Nonetheless, the variation also existed in the definition of APC by MFC affecting ability to differentiate normal from neoplastic PC. To overcome this challenge, the College of American Pathologists in 2014 recommended to include the LOD and the LLOQ for flow-based MRD assays in the final diagnostic report along with the documentation of methods for MRD assay's LOD measurement.[50] Furthermore, the International Clinical Cytometry Society, European Society for Clinical Cell Analysis, and the Euroflow Consortium recommend the harmonized use of different reagents, antibody panels, sample processing protocols, instrument standardization and LOD to improve the sensitivity and reproducibility of MFC-MRD detection in MM.[28,30] Recently, an international harmonized approach for data analysis has been evaluated across seventeen participants from thirteen countries using a consensus gating protocol to reduce inter-laboratory variation in MRD reporting.[51] Taken together the precise definition of LOD and LOQ is mandatory for reliable and reproducible MRD reporting.

Optimal Timing to Assess MRD

There is no data to depict exact frequency of MRD monitoring. In this context, it is important to mention that sustained MRD negativity has survival benefits over MRD negative status documented at single time point. In a recent study, Sharma et al demonstrated that MM patients with MRD negativity had survival benefit at the end of induction chemotherapy or at day 100 post-transplant in terms of PFS ($p = 0.0002$) and overall survival (OS; $p = 0.009$).[52] San-Miguel et al also showed that patients with newly diagnosed

multiple myeloma who achieved sustained MRD negativity lasting more than or equal to 6 and more than or equal to 12 months had deep remission and improved clinical outcomes.[53] Suggested time points for MRD assessment in MM based on literature review are as follows:

A. At first CR—to evaluate the depth of response.
B. Post-therapy—after a predefined number of chemo-cycle
C. Prior to autologous stem cell transplant (ASCT)—to assess transplant eligibility.
D. Post-ASCT—at day 100 (most of the literature on MRD is based on this time point)
E. During maintenance therapy—at periodic intervals to assess duration of response.

Of all these time-points day 100 post-ASCT is the most studied in clinical trials and demonstrated superior survival of MRD-negative patients on day 100 post-ASCT as compared to MRD positive patients. Recently, we too have demonstrated that day 100 post-transplant MRD positive status is associated with poor survival outcome compared to MRD negative status with progressive worsening of both PFS and OS with increase in MRD levels.[35]

Depth of MRD Monitoring

Latest IMWG guidelines define the MFC-based MRD negative status as the absence of phenotypically aberrant clonal PC by NGF on BMA using the Euroflow standard operative procedure (or validated equivalent method) with a minimum sensitivity of 1 in 10^{-5}.[53] Thus, IMWG recommends minimal sensitivity of 10^{-5} or higher for the BMA-based MRD testing. A few publications in the literature correlated the log reduction in MRD assessment with survival where researchers studied MRD level as a continuous variable determined by flow cytometry and demonstrated survival benefits in MM patients per log depletion in MRD.[54,55]Likewise, our group also demonstrated that gradual fall in MRD levels from 0.1 to 0.001% is predictive of superior survival outcomes[35] (►**Fig. 4**). In order to get an optimal sensitivity for clinical relevance more prospective studies needed to define the depths precisely since disease progression and relapse evident in MRD negative subsets as well.

MRD to Make Therapy Decisions

Prognostic value of MRD evaluation in predicting long-term survival has been demonstrated in major clinical trials. A meta-analysis by Munshi et al demonstrated the strong prognostic impact of MRD negativity in MM patients and confirmed the utility of MRD as a relevant surrogate for PFS and OS in MM.[56] However, acceptance of MRD as a surrogate endpoint for treatment requires correlation with PFS and OS benefit in larger cohort of patients in multiple trials.

Future Perspectives

With the growing evidence of MRD as the strong and independent predictor of survival in MM, it is recommended to evaluate MRD more precisely and accurately to benefit the patients in the long run.

Role of Flow Cytometry in Clonal Evolution

Though the phenotype and antigenic variability of malignant PC can be evaluated by flow cytometry, there is limited evidence of the identification of intratumoral heterogeneity by FCMI. Intratumoral heterogeneity can be described as the heterogeneous distribution of genetically distinct MM clones and subclones within a single patient and may complicate diagnostic and prognostic groups posing a significant challenge for disease monitoring. However, FCMI can identify multiple subclones of malignant PC within the same patient based on differential expression of various surface markers on different subpopulations of PC (►**Fig. 5**). This highlights the potential role of FCMI in identifying clonal evolution in MM cases when studied at multiple time points and needs to be explored further in clinical trials to see the impact of these clones on treatment response to explore if a particular subclone is treatment refractory or is responsible for possible relapse.

Emerging Techniques for MRD Evaluation

NGS-based approach for MRD investigation has drawn the attention of researchers in the recent past, however, limited by the costing, labor-intensive technique with analytic expertise. A few studies have also investigated the role of gene expression

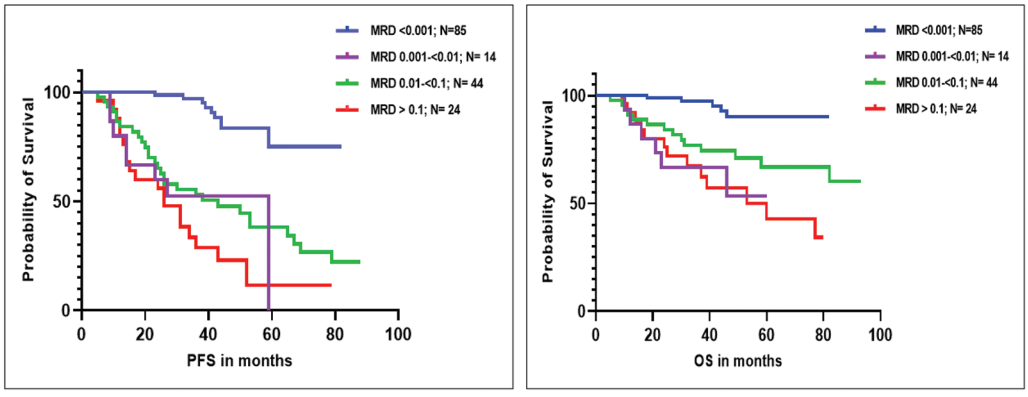

Fig. 4 Kaplan–Meier survival curves displaying sequential improvements in PFS and OS for each log depletion in MRD level. OS, overall survival; MRD, measurable residual disease; PFS, progression free survival.

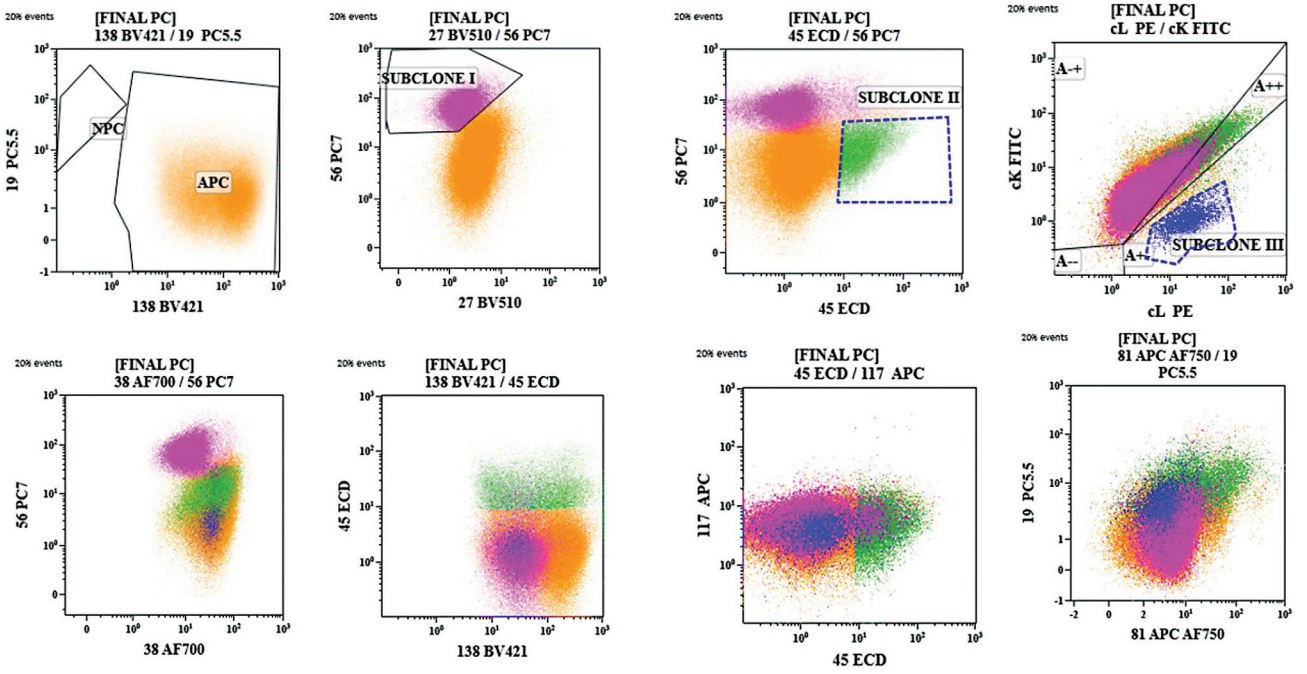

Fig. 5 Flow cytometric dot plots showing multiple subclones of APC (major myeloma clone: orange events; subclone I: magenta events; subclone II: green events; subclone III: blue events) in a case of NDDM. APC, aberrant plasma cell; NDMM, newly diagnosed multiple myeloma.

profiling in myeloma MRD estimation and revealed the role of differentially regulated genetic pathways in MRD landscape of MM.[57,58] Furthermore, considering the patchy nature of disease and spatial heterogeneity in MM, BMA sample may not always be the representative for MRD assessment by MFC or molecular techniques. Nonetheless, in a substantial proportion of extramedullary disease/relapse, BMA is not the ideal sample and may lead to false-negative MRD status. Imaging studies for residual disease estimation may be useful in such scenarios, however, limited by failure to identify the disease present at microscopic/submicroscopic levels. In this regard, utilizing peripheral blood for MRD evaluation has been investigated recently using cell free DNA (cfDNA).[23] In a long-term study on blood based MRD monitoring in MM, cfDNA detection of VDJ rearrangement by ASO-qPCR was used to demonstrate its utility as a prognostic marker.[59]

Conclusion

In conclusion, flow cytometry offers a promising role in MRD estimation in MM. However, it suffers from the inherent challenge of standardization and expertise in analysis. Thus, adoption of a unanimous harmonized approach for MRD analysis by flow cytometry as well as integration of MRD reporting in clinical trials is the need of the hour to utilize MM MRD as a surrogate clinical endpoint.

Authors' Contributions
R.G. and N.D. conceived the study, wrote the manuscript, and approved the final manuscript.

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Conflict of Interest
None declared.

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