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Role of flow cytometric measurable residual disease assessment in multiple myeloma

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Abstract

Despite the high rates of complete response achieved with current treatments, patients with multiple myeloma (MM) continue to relapse due to the presence of minute amounts of residual MM cells. These are referred to as "minimal" or "measurable" residual disease (MRD).

As conventional serological and morphological techniques have become suboptimal for evaluating the depth of response, high sensitivity methods, next-generation flow (NGF) cytometry and next-generation sequencing are recommended in MRD assessment in the bone marrow. Under optimal conditions, these methods can detect one MM cell among 1,000,000 normal cells (a sensitivity of 10^{-6}). Furthermore, imaging techniques, particularly positron emission tomography–computed tomography, have an important role to play in MRD assessment outside of the bone marrow, and alternative blood-based methods for MRD assessment are under investigation. There is a growing consensus that MRD is the most relevant prognostic factor in MM, and achieving a negative MRD status significantly prolongs progression-free survival and overall survival.

This review examines the various methods used to detect MRD, including methodological aspects of NGF. It also presents considerations for implementing MRD as a surrogate biomarker to accelerate drug development and guide MM therapy.

Key words: multiple myeloma, measurable/minimal residual disease, next-generation flow, next-generation sequencing

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Introduction

Multiple myeloma (MM) is the second most common hematological malignancy, accounting for c.10% of all hematological cancers. The annual incidence in Europe is 4.5–6 cases per 100,000 [1]. In Poland, c.1,600 new cases of MM are reported each year [2]. The disease is caused by a proliferating clone of neoplastic plasma cells that destructively affect the bone marrow (BM) microenvironment and, in most cases, secrete a non-functional monoclonal protein (paraprotein, M – protein) into the blood [3].

Advances in diagnostics and risk stratification, and more importantly the increasing availability of new therapies, have improved long-term outcomes for patients with MM [4]. Current treatment regimens using immunomodulatory drugs and second- and third-generation proteasome inhibitors in combination with autologous stem cell transplantation (auto-SCT) are achieving complete responses (CR) in

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up to 70–80% of patients [5–7]. New therapeutic options such as monoclonal and bispecific antibodies or chimeric antigen receptor T-cell (CAR-T) therapy, offer the opportunity to advance treatment in refractory and relapsed disease [8, 9]. The repertoire of therapeutic options is constantly expanding, and the quality of responses achieved will increase as new drugs are used in earlier lines of therapy.

Nevertheless, MM remains an incurable disease for most patients, and the clinical course of MM is characterized by relapses, increasingly short periods of remission, and the development of refractory disease [10].

Improvements in the frequency and quality of responses observed with new drugs and treatment regimens have necessitated the development of more sensitive methods to measure MM clone eradication [11]. Since 2016, the response criteria used to assess treatment efficacy have included deep response categories with measurable/minimal residual disease (MRD) in the bone marrow (BM) aspirate assessment and evaluation of extramedullary disease using imaging techniques. MRD should be assessed by high-sensitivity methods: multiparameter flow cytometry (MFC) or next-generation sequencing (NGS), with a recommended sensitivity of at least 10^{-5} [12]. It has been shown that post-treatment MRD negativity is associated with significantly better progression-free survival (PFS) and overall survival (OS) in newly diagnosed and relapsed/refractory MM patients [13]. In the era of intensive development of modern therapies, the introduction of the MRD criteria has opened up a number of possibilities for the application of this parameter.

In this article, we describe the currently used techniques for MRD testing, including methodological aspects of the flow cytometric method, as well as emerging techniques for improved characterization of residual populations that could be adapted for MRD monitoring in the future. We also discuss the relevance and applicability of MRD testing in clinical trials to determine the potential role of MRD assessment in clinical practice.

Evolution of response criteria in MM

Standardized criteria for assessing the efficacy of anti-myeloma therapies date back to the 1990s, when the prognostic role of achieving a complete response in patients following high-dose chemotherapy and auto-SCT was defined [14, 15]. Since then, in response to progressive improvements in the efficacy of new drugs and patient survival, the International Myeloma Working Group (IMWG) expert panel has updated and defined new categories [12, 16, 17]. These category definitions are based on biochemical test parameters assessing serum and urine M-protein and laboratory methods with varying sensitivity for detecting the degree of BM involvement. CR is defined as undetectable M-protein in serum and urine immunofixation and less than 5% of plasma cells in the BM cytomorphological examination, regardless of their clonality [12]. In contrast, the determinants of 'stringent complete response' (sCR) introduced in 2006 are, in addition to the fulfillment of CR conditions, the normalization of the serum-free immunoglobulin light chain (sFLC) ratio, and the absence of clonal PCs by immunohistochemistry (IHC) or cytometric examination of the BM aspirate using 2–4 markers, the sensitivity of which is estimated to be 10^{-2}–10^{-3} [16].

CR is the primary goal of therapy and its achievement is associated with improved treatment outcomes, including PFS and OS [18]. However, due to its limited sensitivity and the long half-life of the M protein, it does not reflect the true degree of eradication of the tumor clone. In turn, sCR is of limited value in differentiating between patients in CR with different risks of progression [19, 20].

In a retrospective analysis, Cedena et al. found that in a group of patients in CR, obtaining sCR did not identify patients with different PFS (68 vs. 69 months, $p = 0.5$). In contrast, the detection of MRD in patients with sCR with a sensitivity of 10^{-4} (by MFC technique) or 10^{-6} (by NGS technique) was associated with a significantly shorter median PFS compared to the MRD-negative group (for MFC, respectively: PFS 58 months vs. not achieved, $p = 0.04$ and for NGS respectively: PFS 32 months vs. not achieved $p = 0.001$) [20].

The term 'MRD' appeared in the International Myeloma Working Group (IMWG) response criteria as early as 2011, when the categories of immunophenotypic and molecular CR were first introduced, allowing for better risk stratification in an increasing number of patients achieving CR [17]. This required the quantitative assessment of MM cells at the detection level of 10^{-4}–10^{-5} using at least a 4-color MFC and a molecular technique: allele-specific oligonucleotide polymerase chain reaction (ASO-PCR). The sensitivity and specificity of MRD assays have since increased due to advances in cytometry and molecular biology. Second-generation MFC, using eight markers and in most cases achieving a sensitivity of 10^{-5}, have proved to be 30% more effective in detecting MRD than the first-generation MFC, which usually used 4–5 antigens and analyzed 200,000 cells [21]. In the PETHEMA/GEM2010 clinical trial, post-treatment MRD status was not only an independent predictor of time to progression (TTP) [hazard ratio (HR), 2.7; $p = 0.007$] and OS (HR, 3.1; $p = 0.04$), but it was also found that a deeper MM clone reduction overcomes the unfavorable prognosis associated with high-risk cytogenetics and patient age [21]. In subsequent studies, a consistent improvement in PFS and OS outcomes was observed as a function of the logarithmic decrease in the degree of BM involvement by the MM clone [22–24]. This relationship justified efforts to improve existing MRD detection techniques and to search for more sensitive ones.

Table I. Criteria for measurable residual disease (MRD) in multiple myeloma (MM) according to the International Myeloma Working Group (source [12])

All the below require a **complete response** defined as: negative immunofixation on serum and urine and disappearance of any soft tissue plasmacytomas and <5% plasma cells in BM aspirate	
Response criteria	**Definition**
Flow MRD-negative	Absence of phenotypically aberrant clonal plasma cells by NGF in BM aspirate using EuroFlow standard operation procedure for MRD detection in multiple myeloma (or validated equivalent method) with a minimum sensitivity of 1 in 10^5 nucleated cells or higher
Sequencing MRD--negative	Absence of clonal plasma cells by NGS in BM aspirate in which presence of a clone is defined as less than two identical sequencing reads obtained after DNA sequencing using LymphoSIGHT platform (or validated equivalent method)* with a minimum sensitivity of 1 in 10^5 nucleated cells or higher
Imaging-positive MRD--negative	MRD negativity as defined by NGF or NGS plus disappearance of every area of increased tracer uptake found at baseline or a preceding PET-CT or decrease to less mediastinal blood pool SUV or decrease to less than that of surrounding normal tissue
Sustained MRD-negative	MRD negativity in BM (NGF/NGS, or both) and by imaging, confirmed a minimum of one year apart. Subsequent evaluations can be used to further specify the duration of negativity (e.g. MRD-negative at five years)

*ClonoSEQ assay Adaptive Biotech's was approved by Food and Drug Administation in 2019; BM — bone marrow; NGF — next-generation flow; NGS — next-generation sequencing; PET-CT — positron emission tomography–computed tomography; SUV — standardized uptake value

In 2016, another version of the IMWG response criteria was proposed, increasing the recommended sensitivity level of MRD assays (Table I) [12]. MRD-positive status was defined as the persistence or reappearance of clonal PCs in the BM aspirate of patients with CR, assessed with a sensitivity threshold of at least 10^{-5}, which means the need to detect 1 MM cell of among at least 100,000 normal BM cells. Techniques with a sensitivity of 10^{-5}–10^{-6} were considered the reference methods for MRD assessment: next-generation flow (NGF) cytometry with the test protocol developed by the EuroFlow consortium and NGS of immunoglobulin genes performed using the LymphoSIGHT platform (Sequenta/Adaptative) [12, 25, 26]. At the recommended sensitivity threshold, NGF and NGS are considered equivalent techniques; depending on availability, any platform that achieves adequate sensitivity and reproducibility can be used. Due to the heterogeneous nature of BM involvement and the possibility of extramedullary relapse, imaging techniques, particularly positron emission tomography–computed tomography (PET-CT), is a complementary part of assessing a high-quality response in MM [12]. The measure of obtaining a high-quality response with the most favorable prognosis is the category known as sustained MRD-negative, definined as patients with MRD-negative results in BM and imaging tests, confirmed in at least two consecutive assessments within one year [12].

The specificity of the laboratory techniques, and their limitations, significantly affect the sensitivity of the tests, resulting in different limits of MRD detection. In addition, the dependence of the test quality on pre-analytical factors, and the belief that a negative test result does not mean the absence of disease, are the main reasons why the term "measurable residual disease" has been recommended for several years instead of "minimal residual disease".

IMWG — approved methods for MRD assessment

Flow cytometry

MFC, due to its availability, short turnaround time, and relatively low cost, offers the possibility of real-time monitoring of MRD and has the potential to be used in routine clinical practice. MM cells are detected by specific immunophenotypic features that distinguish them from normal/reactive plasma cells. The total population of plasma cells (PCs) in the test sample is determined by the expression of CD38, CD138, and CD45 and parameters determining the size (FSC, forward scatter) and granularity (SSC, side scatter) of the cells. The phenotype of MM cells is determined by abnormal expression patterns of at least two of the most commonly assessed membrane antigens: CD19, CD20, CD27, CD28, CD56, CD81, CD117, or CD200 in conjunction with the intracellular assessment of kappa (cIgκ) and lambda (cIgλ) immunoglobulin light chains [27].

Over the years, several attempts have been made to standardize the method, and recommendations regarding the test procedure have been published [28–31]. A reproducible and validated approach for the highly sensitive assessment of MRD in MM has been proposed by the EuroFlow consortium and defined as 'next-generation flow' (NGF) [25]. The optimized NGF antibody panel contains two 8-color tubes in which ten PCs markers are evaluated: CD38, CD138, CD45, CD19, CD56, CD27, CD81, CD117, cIgκ and cIgλ (Figure 1). This was intended to maximize the likelihood of defining an aberrant PCs population while simultaneously providing important information on sample quality and internal positive and negative control cell populations [25]. Moreover, the multiparametric panels provide valuable information about the tumor microenvironment

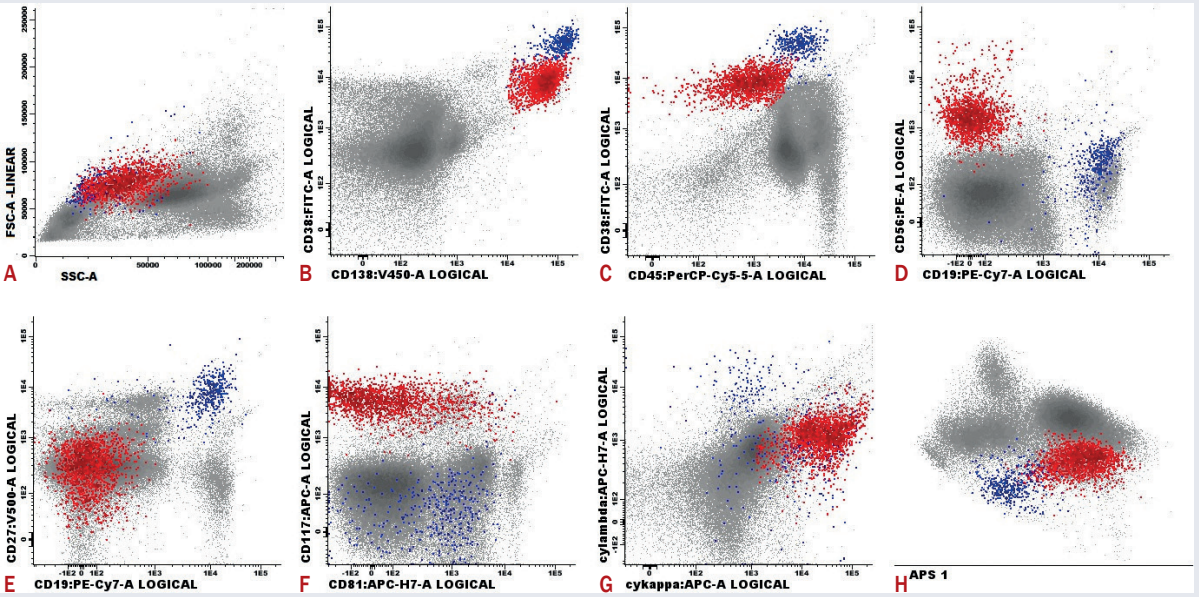

Figure 1A–H. Representative dot plots of analysis of measurable/minimal residual disease assay performed by next-generation flow method using Infinicyt software. Multiple myeloma (MM) cells (red dots) are detected by comparison to normal plasma cells (PCs) (blue dots) and show: high forward scatter (FSC) and medium side scatter (SSC) characteristics, specific high expression of CD138, lower than normal expression of CD38, CD45 and CD27, absence of CD19 and CD81, and aberrant positive expression of CD56 and CD117. Clonal nature of MM cells confirms cytoplasmic kappa light chain restriction. Final plot shows distinct separation of normal PCs and MM cells by automatic population separation (APS) function in Infincyte software. Gray color represents total acquisition events

and the individualized patient immune profile during MRD examination [32, 33].

The simultaneous analysis of at least eight markers is highly specific; thus, detecting PCs immunophenotypic aberrations is possible in all patients. Moreover, a broad antibody panel allows for high sensitivity of MRD assessment without knowledge of the primary antigenic characteristics of MM cells [34]. It is important to note that MM cells can exhibit varying degrees of heterogeneity at the immunophenotypic level. The expression of individual antigens is variable, which may be related to specific molecular alterations and the presence of PC subclones reflecting MM's clonal heterogeneity [35, 36].

In the EuroFlow protocol, the standard **stain-lysis-wash** sample preparation procedure has been replaced by the **lysis-wash-stain-wash** method, in which an appropriate amount of the BM sample is lysed to remove erythrocytes and obtain a suspension of millions of leukocytes in a small volume [25]. NGF requires acquiring and analyzing at least 5 million cells from a test tube (10 mln/test). Considering the losses during preparation, this means that 15–20 million leukocytes must be stained. This guarantees a sensitivity of 4×10^{-6}.

This increases to 2×10^{-6} if we use the special analysis software Infinicyt (Cytognos), which allows us to combine data from two test tubes. This allows for the automatic gating of PCs and identification of the MM cell population, which speeds up the analysis and reduces the risk of subjective evaluation errors. However, at this stage, it will not replace an experienced cytometrist who, when analyzing the MRD examination, must take into account the high immunophenotypic heterogeneity of both MM cells and normal PCs, the presence of MM subclones, and the possibility of modulation of antigen expression after treatment [35–37].

The clinical relevance of the highly sensitive MRD tests was confirmed in a study comparing the NGF approach to the conventional 8-color MFC [25]. 110 BM samples from patients with MM who achieved at least VGPR were evaluated. NGF showed higher sensitivity than 8-color MFC, with 47% versus 34% (p = 0.003) of MRD-positive samples detected. This translated into significantly longer PFS for MRD-negative versus MRD-positive patients (p = 0.01) [25]. The PETHEMA/GEM2014MAIN clinical trial, where the MRD was assessed in 458 patients, confirmed the high sensitivity and efficiency of the MRD NGF method, achieving a sensitivity of $<2 \times 10^{-6}$ in 1% of tested samples, $2 \times \times 10^{-6}$ to $<10^{-5}$ in 88% of samples, $\geq 10^{-5}$ to $<10^{-4}$ in 99.9% of samples, and $\geq 10^{-4}$ in 100% of samples. In only 0.4% of cases was the MRD assessment unreliable due to insufficient quality of the BM sample or technical issues [38].

The sensitivity of MRD assays is significantly affected by sample quality, quantity, and stability. Hemodilution of the BM aspirate sample is the most common pre-analytical

challenge, and is usually due to an incorrect collection procedure. According to current understanding, a patient's MRD status during treatment is the most important prognostic information that can be obtained from the BM aspirate; this is very important to provide high-quality samples for high-sensitivity tests [28]. The first portion of BM from aspiration, not exceeding 2–3 mL, should be collected for MRD studies; further aspiration from the same 'pull' is likely to be hemodilute. Post-acquisition assessment for suboptimal, hemodilute or hypocellular BM samples should be performed, and commented on in the final report. To some extent, hemodilution can be determined cytometricaly by quantifying cell populations that are typically absent in the blood, i.e. precursors of B cells, mast cells and erythroblasts [25]. The MRD report should indicate the potential risk of hemodilution and false negative MRD results in cases with reduced percentages of the cell types mentioned. Reference values for normal BM-associated cell populations have been established [25], although recent studies have shown that their range may vary depending on the time of examination and the type of therapy [39].

It should be noted that the disturbed distribution of the BM cell population may also be the result of impaired hematopoiesis, e.g. due to treatment. Therefore, it is necessary to develop a method with greater specificity to define hemodilution in BM, and the indications for repeated BM aspiration have not yet been clearly defined [39, 40].

According to the recommendations, the laboratory is obliged to determine the sensitivity of the assay obtained in a given test [31]. In particular, the reporting of the limit of detection (LOD) — valid for undetectable MRD, and the lower limit of quantification (LLOQ) — significant for quantitative determinations, are critical parameters for analytical performance. The LOD is defined as the ability of the test to detect MRD at a level that can be reliably distinguished from background noise. The LLOQ is defined as the lowest number or percentage of aberrant PCs that can be reproducibly detected with predetermined bias criteria. These parameters are determined by the identification of at least 20 (for LOD) and 50 (for LLOQ) MM cells and are strictly dependent on the number of BM cells analyzed [31, 41]. As MM MRD assays are highly specialized, the guidance for diagnostic laboratories that wish to perform MM MRD by MFC suggests considering important factors such as the number of MRD tests per year, staff expertise, the fitness of equipment, the availability of a partner laboratory for support and sample exchange, and participation in an external quality control [42].

Caution in the interpretation of single negative MRD results is also warranted by the fact that PCs are underrepresented in BM aspirates, which is particulary evident at diagnosis. This is due to the biology of the disease itself, including remodelling of the extracellular matrix of the BM stroma by the neoplastic process, or the presence of adhesion molecules, e.g. CD56, on the surface of MM cells [43]. False negative MFC results can also be caused by the high sensitivity of PCs in *ex vivo* conditions.

Therefore, it is recommended that the test be performed within 24–48 hours of collection and that appropriate transport conditions be used to maintain a constant temperature. Caution should be exercised in interpreting the CD138 antigen, as it has the greatest expression instability [30].

Daratumumab or isatuximab are anti-CD38 IgG kappa monoclonal antibodies that, combined with standard therapy, improve the quality of response and prolong the survival of patients with relapsed and refractory MM, and are increasingly being used in first-line therapy [44, 45]. It must be emphasized that information about a patient's treatment with immunotherapy is crucial for diagnostic laboratories performing serological, biochemical, or cytometric tests [46, 47]. Anti-CD38 therapy significantly reduces the effectiveness of immunophenotypic detection of PCs with CD38 antigen in MRD assays. The solution may be the use of a multi-epitope CD38 antibody that binds to the antigen site not covered by the therapeutic antibody, other markers such as CD229, CD319, CD54, or the VS38c antibody — which binds to the intracellular protein highly expressed in plasma cells and is tested using the protocol for the evaluation of cIgκ/cIgλ [48, 49].

While the variability in data collection and reporting of results in the context of clinical trials still receives attention [50], it appears to be less and less of an issue in how the test is performed in cytometry laboratories. Published recommendations regarding antibody panel design, sample preparation, data analysis, and finally, validation of the EuroFlow method, have all been important steps towards interlaboratory standardization of MRD testing in MM [25, 30, 31]. The diversity of the procedures regarding the number of cells analyzed, the antibody combinations, the analytical strategies, and reporting has significantly impacted the test sensitivity obtained in different centers [51]. The results of a survey analyzing the method of MRD assessment in MM in Poland showed a high variability of procedures and as much as a 100-fold difference in the sensitivity achieved between different laboratories [52]. Subsequently, harmonizing cytometer parameters and assay protocols in four cytometric laboratories allowed a high, 95%, concordance of results obtained in laboratories, even in samples with very few pathological PCs [53]. This confirms the value of the NGF method and legitimizes standardization activities that ensure consistency in the interpretation of MRD assessment results, which is necessary for multicenter projects.

Since the approval of the NGF standard, several groups have demonstrated alternative antibody panel compositions [54–56] and sample preparation methods for cytometric MRD MM assays [57, 58]. According to the IMWG recommendations, the newly introduced MRD method

should be properly validated by comparison with the results obtained using the reference method. Single 10-color tube antibody combinations have been developed, and studies comparing performance and reliability have shown a 95–98% agreement with the results obtained using the NGF method [54, 55]. While the advantages of the single tube method include lower cost due to reduced labor, reagents, and processing time, it should be noted that high agreement was found mostly up to a sensitivity threshold of 10^{-5} (0.001%). The two-tube method has been considered more robust because of the higher number of measurable cells, and the confirmatory value of the second tube for small populations of cells suspicious of MRD found in the first tube [54–56]. It has also been emphasized that including cytoplasmic kappa/lambda light chain markers in the 10-color panel significantly increases the assay's specificity [56].

Next-generation sequencing

Molecular techniques can reliably detect MM MRD since they provide precise disease measurements with high sensitivity. NGS has replaced another molecular method, ASO-quantitative (qPCR), because of its higher sensitivity, lower workload, and ability to be used in a greater percentage of patients with MM (>90% for NGS vs. <70% for ASO-qPCR) [26, 59]. This platform uses specific primers to amplify and sequence immunoglobulin gene segments with locus-specific primers for IgH (VDJ), IgH (DJ), or IgK rearrangements. After amplification, the immunoglobulin gene DNA is sequenced to determine the frequency of different clones. Importantly, MRD monitoring requires the identification of a patient-specific sequence from a pre-treatment sample, and the method has a slightly lower applicability than NGF (c.95% vs. 100%), as in some patients the dominant clonal sequence of MM cells cannot be detected in diagnostic samples [60].

In recent years, several NGS platforms for MRD detection in MM have been tested, achieving high sensitivity in the detection of MRD at the level of 10^{-5}–10^{-6} [61] or even 10^{-7} [62]. The ClonoSeq assay (Adaptive Biotechnologies, Seattle, WA, USA) was the first to be approved by the US Food and Drug Administration (FDA) and is currently the most frequently used for disease assessment in MM patients [63]. Following the promising results of Martinez-Lopez et al. [26], subsequent studies have confirmed the prognostic value of MRD assessment by NGS [61, 62, 64]. Perrot et al. confirmed these important findings in a larger series of MM patients enrolled in the IFM2009 clinical trial. PFS and OS were significantly prolonged in NGS MRD-negative vs. MRD-positive patients at pre- and post-maintenance timepoints [64]. Studies comparing MRD results from NGS and MFC assays at a sensitivity level of 10^{-5} showed an 83–85% concordance between the two techniques and 78% at a 10^{-6} sensitivity level [65, 66]. This suggests that NGS is more likely to reach a sensitivity threshold of 10^{-6} than NGF, but the problem with this type of study is often differences in sample quality. Similar to NGF, the sensitivity of the NGS MRD test is highly dependent on the quality of the BM aspirate. Nonetheless, NGS requires fewer cells for the assay (approx. 3 million vs. 20 million for NGF) [67]. However, unlike cytometry, the NGS method does not allow for sample quality assessment. The feasibility of NGS is limited by its high cost, long turnaround time, and high degree of expertise required. The advantage of this approach is that it can be applied retrospectively to stored material, including cryopreserved cells and archival BM slides. Moreover, the specificity of NGS allows for tracking clonal heterogeneity and the dynamics of molecular changes that occur during the disease. The methods used to assess MRD in BM, and their advantages and disadvantages, are set out in Table II.

Imaging MRD assessment

MM distribution is often heterogeneous, and imaging can be used to complement MRD detection at a single site. According to the IMWG criteria, additional MRD assessment outside the BM is mandatory to define the deepest possible response, and PET-CT is the current optimal technique [12]. The 'imaging plus MRD-negative CR' category further stratifies patients, and normalization of the PET-CT image after treatment correlates with longer PFS [68, 69]. It has been shown that combining MFC MRD and imaging improves outcome prediction, with double-negative and double-positive features defining groups with excellent and dismal PFS, respectively [70]. The presence of focal (metabolically active) areas of fluoro-2-deoxyglucose (FDG) uptake after induction therapy is associated with a higher risk of disease relapse, even in patients who achieved MRD-negative status in BM assessment. Moreau et al. [68] found a concordance of almost 62% between MFC MRD in BM and PET-CT negativity after consolidation, with 6.8% of patients showing PET-CT positivity and a negative MRD result. In the PETHEMA/GEM study, half of the patients with progression confirmed by PET-CT had no serum M protein or BM involvement.

These observations highlight the need to combine NGF or NGS with PET-CT to monitor the effectiveness of treatment, especially in patients with extramedullary and multifocal diseases [37, 70].

Importance and clinical application of MRD assessment in MM

The prognostic significance of MRD in MM was first emphasized in two publications in 2002 by a Spanish and British research groups evaluating the efficacy of auto-SCT in MM therapy [71, 72]. Three months after auto-SCT, with MFC sensitivity of 10^{-4}, MRD was detected in 30% [71] and 60% [72] of patients with negative immunofixation. Both the absence of clonal plasma cells (PCs), and more

Table II. Characteristics of techniques for monitoring multiple myeloma minimal/measurable residual disease in bone marrow (BM)

Variable	NGF	NGS
Method	Clonal cells are identified by their distinct immuno-phenotypic pattern vs. normal plasma cells	Specific immunoglobulin rearrangements are identified and detected by comparison with baseline sample
Reference platform	EuroFlow standardized 2-tube 8-color approach	Lymphosight, CloneSeq
Applicability	~100%	>90%
Baseline sample	Important but not mandatory	Mandatory
Number of cells required	10 million cells/tube	2–3 million cells/20 µg DNA
Sensitivity	10^{-5}–10^{-6}	10^{-5}–10^{-6}
Sample processing	Within 24–48 h Requires fresh sample	Fresh and stored samples can be used
Time required	3–4 h	1–2 weeks
Sample quality control	Concurrent with BM analysis	Not possible
Clonal evolution	Not evaluable	Evaluable
Support required	Automated software; expert flow cytometrist	Bioinformatics support

NGF — next-generation flow; NGS — next-generation sequencing

than 30% of normal PCs in the total plasma cell population, correlated with longer PFS (p = 0.04 and p = 0.02, respectively) [71, 72]. Subsequent analyses using more sensitive MFC, ASO-PCR, and NGS techniques have shown that a deeper response correlates with improved PFS and OS, suggesting that the goal of treating patients should be to achieve the deepest possible eradication of the MM clone [64, 73, 74].

The abundant scientific evidence of the prognostic value of MRD in MM has been summarized in meta-analyses [13, 75–77]. In an analysis of 1,273 patients from 14 studies, undetectable MRD was associated with a significant increase in PFS [hazard ratio (HR) 0.41; 95% CI: 0.36–0.48; p <0.0001]. The effect on OS was assessed in 1,100 patients included in 10 studies, which showed a clear benefit from achieving MRD-negative status (HR 0.57; 95% CI: 0.46–0.71; p <0.0001) [75]. Lahuerta et al. [76], in an analysis of 609 patients from three Spanish clinical trials, demonstrated the superiority of MRD over conventional CR, as MRD-detected CR patients had similar survival to MRD-positive patients who did not achieve CR. This relationship was confirmed in patients eligible and ineligible for auto-SCT, and in subgroups stratified by disease stage (ISS, International Staging System) and cytogenetic risk profile [76]. The recent meta-analysis by Munshi et al. [13] reviewed data from up to 93 publications from 45 studies, including 8,098 patients, and has confirmed the significance and strong prognostic value of MRD in a heterogeneous cohort of patients from different prognostic groups. The benefit of a negative MRD result was evident regardless of treatment, cytogenetic risk, MRD assessment method, or sensitivity level. As expected, the greatest benefit in terms of PFS and OS was observed in patients who had a negative MRD result at a sensitivity level of <10^{-6} [13]. Furthermore, the absence of MRD had a prognostic value in both CR and in very good partial remission (VGPR) patients, which seems to be particularly important in assessing the effectiveness of new immunotherapies that induce rapid and deep responses [13, 76].

While the standard endpoints of PFS and OS provide the most conclusive evidence of treatment efficacy, recent advances in MM treatment have significantly prolonged patient survival, making prospective clinical trials both lengthy and costly. Therefore, the absence of MRD with a sensitivity of 10^{-5} or even more informative at the level of 10^{-6}, is considered as a surrogate endpoint in clinical trials, also due to its speed of reading and its applicability in various clinical scenarios [78]. Data from these meta-analyses, MFC standardization, and FDA approval of the NGS ClonoSEQ platform may contribute to the final acceptance of MRD as a regulatory endpoint in clinical trials aimed at drug approval and those determining the role of MRD testing in routine clinical practice. Ongoing and future clinical trials using MRD as an endpoint would help assess the efficacy of new treatment regimens and, thus, may determine the validity of auto-SCT after four-agent induction [79] or determine the duration of maintenance therapy. Pawlyn et al. observed, using MFC with a sensitivity of 0.004%, that for patients who were MRD-negative after auto-SCT, the PFS advantage of maintenance lenalidomide diminishes beyond three years, compared to beyond 4–5 years in patients who are MRD-positive [80]. To facilitate the design, conduct, and interpretation of clinical trials, an international panel of experts has formulated recommendations regarding the type of MM studies that should include MRD measurement, recommended assessment timepoints, and expected analytical validation for the MRD tests, and recommendations for the reporting of results [41].

In addition to achieving MRD-negativity, an important aspect of therapy is the maintenance and attainment of a sustained MRD-negativity response [81, 82]. Standardized and sensitive MRD testing can provide more information relevant to understanding disease biology and assessing the likelihood of relapse when performed sequentially at multiple timepoints.

Gu et al. [83] monitored 104 patients with MM after induction and 3, 6, 9, 12, 18 and 24 months after auto-SCT with NGF. Patients with undetectable MRD after induction and throughout the post-transplant follow-up had the best prognosis, with PFS and OS similar to those who achieved MRD-negative status only after auto-SCT [83]. In MRD-negative patients after induction, the reappearance of MRD within 24 months after auto-SCT was significantly correlated with a shorter OS compared to the persistently MRD-negative group (35.2 ± 18.6 months vs. not reached), supporting the validity of long-term MRD monitoring. According to the authors, the optimal time for MRD assessment should include the post-induction period and three and 24 months after transplantation. Monitoring MRD-negative patients every six months would allow early detection of disease progression [83]. A recently published long-term follow-up study show that MRD conversion is associated with a high risk of biochemical or clinical relapse and is preceded by a median of 1.0 year (range 0–4.9 years) [84]. Similar results were presented by Schmitz et al. [85], who analyzed the dynamics of MRD quantitative changes in 20 CR/sCR patients. Increasing MRD levels were observed in six cases. They preceded biochemical changes (abnormal FLC ratio and positive electrophoresis) and clinical progression by a mean of 5.5 and 12.6 months, respectively, with the MM doubling mean time of 1.8 months [95% confidence interval (CI): 1.4–2.3 months] [85]. However, about 27% of patients with MRD resurgence can never experience clinical relapse [84]. Rodríguez-Otero et al. [86] found that long-term survival among patients with persistently MRD-positive disease may be explained by an 'MGUS-like' immunophenotypic signature in the BM at diagnosis defined by the relative frequency of BM PCs plus the percentage of clonal and normal PCs within the whole BM PC compartment.

The rate of both eradication and growth of the tumor clone at relapse can be influenced by a number of factors, including those that stratify patients into risk groups [37, 87]. In the Myeloma IX trial, regardless of baseline cytogenetic risk, the absence of MRD at 100 days after auto-SCT was associated with improved PFS (p <0.001) and OS (p = 0.0183), but median PFS was three times longer in the standard cytogenetic risk MRD-negative group compared to the high-risk MRD-negative group (defined as gain(1q), del(1p32), t(4;14), t(14;20), t(14;16) and del(17p) [88]. Also, in patients with persistent MRD, regardless of logarithmic levels, the presence of high-risk cytogenetic abnormalities conferred poorer outcomes [76, 87]. Other studies have reported that high-risk patients who achieve MRD-negative status at the level of 10^{-5} or 10^{-6} after effective therapies have comparable PFS and OS to standard-risk patients [21, 62]. The factors identified by the cytogenetic analysis and the baseline stratification of patients have a significant impact on the prognosis at the time of diagnosis and during disease progression in the MRD-negative group [38]. The ability to identify patients with the deepest responses may optimize the existing risk assessment tools for MM patients.

Risk stratification may need to be reassessed after treatment, as patients with an adverse prognosis can shift into a favorable one after achieving and maintaining deep responses after intensive therapy [38]. Therefore, MRD testing offers the possibility of a better prognosis, dynamic risk assessment, and modification during the course of the disease, but always in the context of risk factors from the moment of diagnosis and earlier treatment [89].

Tracking disease kinetics by numerical or logarithmic changes in the MRD, even at such low tumor weights, may provide greater information resolution. In a prospective study, Diamond et al. evaluated the dynamics of changes in MRD status based on 340 MFC MRD studies performed over five years in 103 patients treated during lenalidomide maintenance therapy [74]. Patients who maintained an MRD-negative response had no disease progression at a median follow-up of 19.8 months. Interestingly, patients who lost the MRD-negative response were more likely to have disease progression than both patients with persistently negative MRD (p <0.0001) and patients with persistently positive MRD (p = 0.015) [74]. Study results by Alonso et al. [90] confirmed the role of lenalidomide maintenance in stabilizing the response and improving its quality. Sequential annual MRD assessments showed that both achieving MRD negativity and gradually decreasing MRD levels alone significantly prolonged PFS [90]. In a recently published study, Paiva et al. [91] assessed the importance of serial monitoring. They examined the dynamics of MRD measured by NGF in 1,362 patients after induction and during the maintenance phase [91]. MRD-negative patients at the end of induction had a median PFS of 38.6 months, compared to 15.6 months for those with MRD-positive result in BM. At the time of evaluation, 9.5% of patients had converted from MRD-negative to MRD-positive, which was associated with worse PFS, similar to patients who were MRD-positive at every timepoint (2-year PFS rate of c.30%). 5.1% of MRD-positive patients achieved MRD negativity and PFS similar to that of the MRD-negative group (2-year PFS rate of 75%) [91]. These observations highlight the importance of sequential MRD monitoring, which may provide a more accurate assessment of prognosis than measurement at a single timepoint. This may indicate the value of the therapy used and distinguish subgroups of patients with different prognoses. The authors also point to the possibility of early relapse warning and the need to implement anti-relapse treatment [90, 91].

The prognostic significance of a deep MRD-negative response is beyond doubt. Even so, the predictive value, and thus the role, of MRD assessment in routine clinical management has not yet been determined. The available preliminary data suggests the benefits of treatment tailored to the response status, and MRD status can be incorporated into the clinical decision-making process at various timepoints, e.g. to determine the duration of induction therapy [92], the validity of the auto-SCT procedure given the availability of effective induction regimens [93], or the intensity and continuation of maintenance therapy [94]. As one of the first, Korde et al. [92] published the results of a study in which the number of cycles of induction therapy with carfilzomib, lenalidomide, and dexamethasone (KRd) was individualized based on MRD status. In the MASTER trial, patients received daratumumab, carfilzomib, lenalidomide, and dexamethasone (Dara-KRd) induction, auto-SCT, and Dara-KRd consolidation, according to MRD status. MRD was assessed by NGS, and patients with two consecutive MRD-negative assessments remained in follow-up without treatment. The 2-year PFS rate in the observation group was 87%, and the risk of relapse within 12 months after treatment discontinuation was significantly higher in patients with a higher cytogenetic risk [94]. Martinez-Lopez et al. [95] published the results of a retrospective analysis of survival in patients monitored with MRD during first-line therapy. Treatment modification based on MRD results (treatment discontinuation, intensification, or new therapy) was performed in 67 patients, resulting in longer PFS than in patients who did not change therapy (mean PFS 104 vs. 62 months, $p = 0.005$). In patients with at least one MRD negative result during maintenance therapy, discontinuation versus continuation did not change PFS ($p = 0.1$). However, in patients who were MRD-positive during maintenance, intensification or therapy change resulted in a better PFS than patients with no therapy adjustments (mean PFS not achieved vs. 39 months, $p = 0.02$) [95].

Several clinical trials are investigating therapeutic strategies based on MRD status (Table III) [96]. The randomized EQUATE study (NCT04566328) will evaluate the effectiveness of intensifying first-line therapy in patients with a positive MRD result after induction. In turn, the DRAMMATIC (NCT04071457) trial may answer whether maintenance therapy can be safely discontinued in patients with persistently negative MRD. The REMNANT study (NCT04513639) will compare the effectiveness of carfilzomib, dexamethasone, and daratumumab in treating MM relapse, defined as the appearance of MRD versus progression of MM defined by IMWG criteria. The Polish Myeloma Consortium's PREDATOR clinical trial (NCT03697655) will evaluate the role of preemptive daratumumab therapy in biochemical relapse or MM progression defined as MRD reaperance measured in BM with a sensitivity of 10^{-5}.

Peripheral blood techniques for MRD assessment

The focal nature of the bone marrow infiltration, the clonal evolution of MM over time, the possibility of recurrent extramedullary lesions, and the invasiveness of the procedure of regular biopsies, all mean that the optimal monitoring scheme and other methods and techniques to obtain complete information about the actual degree of eradication of the MM clone is still being sought.

An alternative approach to BM testing may be **liquid biopsy** — a diagnostic technique that identifies and analyzes circulating tumor plasma cells (CTPC) or cell-free DNA (cfDNA) in peripheral blood. Both CTPC and cfDNA are currently being investigated for quantitative and qualitative characterization of the tumor genome and as a non-invasive monitoring of MM therapy [97]. CTPCs are released from the primary tumor or metastatic sites into the bloodstream and are responsible for dissemination and extramedullary disease. CfDNA consists of degraded DNA fragments released into the circulation from tumor cells and is molecularly distinct in total extracellular DNA [98]. It has been demonstrated that CTPC can be detected in up to 80–90% of newly diagnosed patients and even in 100% of patients at MM relapse [99, 100]. Several studies have confirmed that detectable CTPC at diagnosis, post-treatment, and pre/post-auto-SCT is an unfavorable prognostic factor for therapeutic response and progression, regardless of the ISS/Revised Multiple Myeloma International Staging System (R-ISS) stage and high-risk cytogenetics [101]. Furthermore, it has been suggested that detecting ≥0.01% CTPC may be a new risk factor in novel staging systems for patients with transplant-eligible MM [99]. Moreover, the results of a study by Garcés et al. [99] showed that this adverse effect on PFS can be overcome by effective treatment and achieving an MRD-negative response in BM. Genomic characterization showed a high concordance of mutations detected in CTPCs and paired BM samples; however, some mutations were only detected in blood, indicating that CTPCs represent a more complete picture of disease burden than cells from BM samples obtained from only one region [102]. In the context of MRD testing, a higher degree of sensitivity is needed, and even with next-generation techniques, peripheral blood assessment appears to be significantly less sensitive than BM-based assays. Sanoja-Flores et al. [103] reported that MRD was present in 17% of patients in CR by detection of CTPC and identified a subgroup of patients with significantly shorter PFS. However, in a significant percentage of patients (40–56%) with a positive MRD result in BM, CTPC/ctDNA in the blood may be undetectable. In turn, MRD has been found in the BM in 88–100% of cases with CTPC present in the blood [103, 104].

These observations suggest that persistently positive MRD in the blood may reflect positive BM MRD and avoid

Table III. Selected trials with measurable/minimal residual disease (MRD) adapted treatment strategy in multiple myeloma (MM) (source [96])

Study ID	Title	Phase/ /planned population	Estimated study completion date	MRD methodology/ /sensitivity	Point of MRD-driven decisions	Brief outline	Primary endpoint
PREDATOR-MRD NCT03697655	Pre-emptive Daratumumab Therapy of Minimal Residual Disease Reappearance or Biochemical Relapse in Multiple Myeloma (PREDATOR)	II 274	July 2024	NGF 10^{-5}	At MRD relapse (loss of MRD-negativity)	Patients with loss of previously attained MRD negativity (observation up to 73 weeks) will be given daratumumab immediately vs. standard of care	Event--free survival (EFS)
MRD-STOP NCT04108624	A Multimodality Approach to Minimal Residual Disease Detection to Guide Post-Transplant Maintenance Therapy in Multiple Myeloma (MRD2STOP)	NA 56	December 1, 2024	NGF 10^{-5} NGS $\geq 10^{-6}$ Blood assays	MRD-negative status after at least one year of maintenance	Patients will undergo discontinuation of maintenance therapy if they are MRD negative by multiple modalities (PET-CT, NGF and NGS) after receiving at least one year of maintenance therapy	MRD conversion date
CONPET NCT03314636	Intensified Treatment With Carfilzomib in Myeloma Patients Still PET--positive After First Line Treatment (CONPET)	II 50	March 2025	PET-CT NGF 10^{-5}	PET-positive after a standard first-line treatment	Patients who are PET negative will be excluded from treatment; those who are PET positive will be given KRd	PET conversion rate
AURIGA NCT03901963	A Randomized Study of Daratumumab Plus Lenalidomide Versus Lenalidomide Alone as Maintenance Treatment in Patients With Newly Diagnosed Multiple Myeloma Who Are Minimal Residual Disease Positive After Frontline Autologous Stem Cell Transplant	III 214	May 29, 2026	NGS 10^{-5}	MRD-positive status after auto-SCT	Evaluation of conversion rate of MRD negativity following addition of daratumumab to lenalidomide relative to lenalidomide alone, when administered as maintenance treatment to patients who are MRD positive after auto-SCT	MRD negativity
NCT04140162	Phase 2 Study With Minimal Residual Disease (MRD) Driven Adaptive Strategy in Treatment for Newly Diagnosed Multiple Myeloma (MM) With Upfront Daratumumab--based Therapy	II 50	October 2026	NGS/MFC 10^{-5}	MRD-positive status after induction	Trial will test whether combination of DaraRd as induction therapy, followed by DRVd consolidation therapy if needed, will result in more patients achieving MRD-negative status, relative to standard of care. Consolidation therapy will be administered only to MRD-positive patients after induction	MRD negativity after induction or, if still MRD--positive, after consolidation

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Table III (cont.). Selected trials with measurable/minimal residual disease (MRD) adapted treatment strategy in multiple myeloma (MM) (source [96])

Study ID	Title	Phase/ /planned population	Estimated study completion date	MRD methodology/ /sensitivity	Point of MRD-driven decisions	Brief outline	Primary endpoint
EQUATE NCT04566328	Testing Use of Combination Therapy in Adult Patients With Newly Diagnosed Multiple Myeloma, EQUATE Trial	III 1,450	December 31, 2027	NGS 10^{-6}	Positive MRD result after induction	DaraRD induction followed by addition of bortezomib to DaraRd for consolidation treatment in MRD-positive patients after induction	Consolidation OS
REMNANT NCT04513639	Relapse From MRD Negativity as Indication for Treatment (REMNANT)	III 176	June 1, 2032	NGF 10^{-5}	Upon MRD relapse (loss of MRD negativity)	Randomization to receive second-line treatment (KRd) either at loss of previously attained MRD negativity or at progressive disease, as per IMWG criteria. Study will evaluate whether treating MRD relapse after first line treatment prolongs PFS and OS versus treating relapse at progressive disease	PFS, OS, MRD negativity 30––45 days post consolidation
DRAMMATIC/ /S1803 NCT04071457	Lenalidomide 6 Daratumumab/ /rHuPh20 as Post–ASCT Maintenance for MM w/MRD to Direct Therapy Duration (DRAMMATIC)	III 1,100	July 1, 2040	NGS 10^{-6}	After two years of maintenance (lenalidomide ± ± Dara)	After two years of maintenance, MRD positive patients continue assigned treatment. MRD--negative patients are randomized to continue/discontinue therapy	OS

NGF — next generation flow; NGS — next generation sequencing; PET-CT — positron emission tomography–computed tomography; OS — overall survival; PFS — progression-free survival; auto-SCT — autologous stem cell transplantation; DaraRd — daratumumab, lenalidomide, dexamethasone; DRVd — daratumumab, lenalidomide, bortezomib, dexamethasone; KRd — carfilzomib, lenalidomide, dexamethasone; IMWG — International Myeloma Working Group

invasive BM assessment. Further studies at different treatment timepoints and using a more sensitive methodology (e.g. with immunomagnetic enrichment) would help clarify the role of CTPC assessment in MM prognosis [105].

Mass spectrometry (MS) methods are emerging as a promising new approach for more sensitive detection and monitoring of paraprotein levels in serum [106]. The basis of the MS method is the unique sequence of the antigen binding region, called the 'complementarity determining region' (CDR) of the immunoglobulin. The CDR amino acid sequence is specific for the MM clone. This gives the immunoglobulin a specific isoelectric point (the basis of the electrophoresis method) and mass (the basis of the M-protein detection by MS). Efforts to optimize M protein detection by MS have resulted in two methods varying in analytical sensitivity: matrix-assisted laser desorption/ionization--time-of-flight mass spectrometry (MALDI-TOF-MS), and liquid chromatography-mass spectrometry (LC-MS) [107]. MS techniques can detect and quantify M-protein with a detection limit approximately 100 times lower than immunofixation, translating to concentration ranges of 0.05 to 0.001 g/L [107]. Published data suggests that MS should be considered as part of a multidimensional approach to MRD assessment. Compared to BM NGF, MS in blood displays a fair degree of concordance and is associated with a comparable prognostic value [108]. Eveillard et al. [108] compared the performance of MALDI-TOF-MS to the MRD MFC 10-color single-tube method. Their study demonstrated that the results of MS were concordant with the MFC MRD in BM for 44/71 (62%) patients (p = 0.342). Of the 27 discordant cases, 17 were detectable only by MALDI-TOF MS, and 10 were detectable only by MFC MRD [108].

These results suggest that MALDI-TOF-MS adds value to BM-based MRD testing and is more specific for early detection of relapse than electrophoretic methods. MS could be used as a screening method for MRD testing in patients whose disease is not detectable by immunofixation (IFE) and sFLC testing [107]. A negative MS result could indicate BM aspiration to confirm MRD-negative status. The use of MS in MRD monitoring is currently limited to the research community, nevertheless the IMWG Mass Spectrometry Committee endorses the detection of M-proteins by MS (MALDI-TOF method) as an alternative to IFE and for distinguishing residual M-protein from therapeutic monoclonal antibodies for clinical practice, and for accurate interpretation and determination of complete response in clinical trials [107].

Conclusions

Achieving MRD negativity is one of the strongest prognostic factors in MM, independent of disease status (newly diagnosed or relapsed), cytogenetic risk, MRD assessment, or the sensitivity method achieved. A negative MRD result, and especially sustained MRD negativity, seems to be more important than the treatment used. Flow cytometry and molecular methods guarantee high sensitivity. However, each method in its current form has its limitations, and the most important of these seems to be the limited representativeness of the BM samples. Imaging assessment techniques and new techniques for evaluating peripheral blood complicate the harmonization of MRD evaluation and require further research, but may prove essential for a comprehensive evaluation of a patient's status.

MRD has been incorporated into numerous clinical trials to compare different treatment approaches, adapt therapy intensities according to MRD status, determine maintenance duration, or introduce early intervention strategies. An important issue remains the determination of the frequency of MRD testing, assessing the likelihood and interpretation of false-positive and false-negative results, and combining different evaluation techniques and additional prognostic biomarkers to supplement the MRD results.

This will require a great deal of analysis and application of the MRD parameter in different clinical contexts, but nonetheless offers an unprecedented opportunity to use MRD assessment to optimize and personalize therapeutic strategies in MM.

Authors' contributions

AK — conceptualization, literature analysis, original draft preparation, review and editing. BP — review and editing. KJ — conceptualization, review and editing. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments and uniform requirements for manuscripts submitted to biomedical journals.

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