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INVITED REVIEW

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Advances in minimal residual disease monitoring in multiple myeloma

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ABSTRACT

Multiple myeloma (MM) is characterized by the clonal expansion of plasma cells and the excretion of a monoclonal immunoglobulin (M-protein), or fragments thereof. This biomarker plays a key role in the diagnosis and monitoring of MM. Although there is currently no cure for MM, novel treatment modalities such as bispecific antibodies and CAR T-cell therapies have led to substantial improvement in survival. With the introduction of several classes of effective drugs, an increasing percentage of patients achieve a complete response. This poses new challenges to traditional electrophoretic and immunochemical M-protein diagnostics because these methods lack sensitivity to monitor minimal residual disease (MRD). In 2016, the International Myeloma Working Group (IMWG) expanded their disease response criteria with bone marrow-based MRD assessment using flow cytometry or next-generation sequencing in combination with imaging-based disease monitoring of extramedullary disease. MRD status is an important independent prognostic marker and its potential as a surrogate endpoint for progression-free survival is currently being studied. In addition, numerous clinical trials are investigating the added clinical value of MRD-guided therapy decisions in individual patients. Because of these novel clinical applications, repeated MRD evaluation is becoming common practice in clinical trials as well as in the management of patients outside clinical trials. In response to this, novel mass spectrometric methods that have been developed for blood-based MRD monitoring represent attractive minimally invasive alternatives to bone marrow-based MRD evaluation. This paves the way for dynamic MRD monitoring to allow the detection of early disease relapse, which may prove to be a crucial factor in facilitating future clinical implementation of MRD-guided therapy. This review provides an overview of state-of-the-art of MRD monitoring, describes new developments and applications of blood-based MRD monitoring, and suggests future directions for its successful integration into the clinical management of MM patients.

Abbreviations: CR: complete response; CT: computed tomography; CTC: circulating tumor cells; DIA: data independent analysis; FDA: Food and Drug Administration; FDG: 2-[(18)F] fluoro-2-deoxy-D-glucose; FLC: free light chain; IMWG: International Myeloma Working Group; LC: liquid chromatography; LLOD: lower limit of detection; m/z: mass to charge ratio; MALDI-TOF: matrix-assisted laser desorption/ionization-time of flight; MM: multiple myeloma; MRD: minimal residual disease; MS: mass spectrometry; MS/MS: tandem mass spectrometry; MS-MRD: mass spectrometry - minimal residual disease; NGF: next generation flow cytometry; NGS: next generation sequencing; PET: positron emission tomography; PFS: progression free survival; PRM: parallel reaction monitoring; SIL: stable isotope labeled; SPEP: serum protein electrophoresis; SRM: single reaction monitoring; t-mAb: monoclonal antibody therapy; UPEP: urine protein electrophoresis

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Introduction

Multiple myeloma (MM), the second most common hematological malignancy, is characterized by the clonal expansion of plasma cells in the bone marrow that produce a monoclonal immunoglobulin (M-protein), or fragments thereof [1]. Electrophoretic M-protein diagnostics combined with serum free light chain (FLC) analysis are the gold standard for monitoring MM disease activity [2,3]. In the past decade, the life expectancy of MM patients has improved substantially because of the introduction of new effective therapies. With modern multimodal therapy regimens

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such as those studied in the CASSIOPEIA and GRIFFIN trials, more than 70% of MM patients achieved a complete response (CR, no detectable evidence of M-protein in serum and urine by using electrophoretic methods and <5% plasma cells in bone marrow) during first-line therapy, which translated into improved survival of MM patients [4,5]. Despite the greatly improved rate and depth of response, MM remains an incurable disease for the majority of patients, with periods of remission followed by relapse and eventual development of multidrug-resistant disease [6]. Given the high number of patients who reach a complete response, major efforts have been made to measure minimal residual disease (MRD) for response evaluation. The International Myeloma Working Group (IMWG) has defined consensus criteria to identify responses beyond conventionally defined CR that allows uniform reporting on MRD status [3]. Bone marrow-based techniques that are used for MRD testing are next-generation sequencing (NGS), next-generation flow cytometry (NGF), and allele-specific oligonucleotide-quantitative polymerase chain reaction [7,8]. These can be combined with imaging techniques to detect extramedullary disease. Not only does MRD evaluation provide a uniform framework for MRD reporting and a potential endpoint for clinical trials, but also it provides a powerful tool for disease prognostication [9]. Recent studies have shown a strong correlation between MRD negativity (no detectable clonal plasma cells in the body after treatment) in patients with CR and prolonged progression-free survival (PFS) [10–14]. Another reason for the increased interest in novel methodologies to measure MRD is the numerous prospective trials in which the added clinical value of MRD is being studied for response-driven treatment decisions (www.clinicaltrials.gov). Currently, approximately 20 clinical trials studying MRD-guided therapy have been registered. Recently the first results of two clinical trials were published. In the MASTER trial, NGS was used to assess MRD status at fixed time points in a cohort of 123 newly diagnosed MM patients. Although this was a single-arm trial, the PFS data suggested that standard-risk and high-risk patients benefited more than ultra-high-risk patients (defined by the presence of cytogenetic abnormalities: 0 (standard risk); 1 (high-risk); 2+ (ultra-high-risk)) from such a therapy-free interval, because the ultra-high-risk patients would most likely need continuous therapy to prevent disease progression [15]. In the GEM2012menos65 trial, patients with positive MRD at two years after autologous hematopoietic stem cell transplantation received lenalidomide/dexamethasone for three years, while MRD-negative patients were not given any additional therapy. Despite the additional three years of maintenance therapy, MRD-positive patients had higher relapse rates compared to MRD-negative patients [16]. This data suggested that discontinuation of maintenance therapy for MRD-negative patients may be beneficial to prevent toxicities while the response is preserved. However, randomized trials comparing the continuation and discontinuation of maintenance therapy based on MRD outcome are necessary to assess the value of MRD-guided therapy. Because MRD-guided therapy relies on periodic MRD monitoring, there is an urgent clinical need for ultra-sensitive techniques such as mass spectrometry (MS) that allow evaluation of MRD (MS-MRD, mass spectrometry - minimal residual disease) in blood as a less invasive, patient-friendly alternative for MRD evaluation performed on bone marrow [17,18]. This review provides a comprehensive overview of the available methods to monitor disease activity in MM patients and focuses on novel ultra-sensitive blood-based MS-MRD methods to detect and quantify M-protein. The advantages and disadvantages of blood-based MS-MRD, the possible complementary value of MS-MRD in the management of MM patients, and the steps that still need to be taken to realize the implementation of blood-based MS-MRD evaluation in clinical practice are discussed.

Methods to monitor myeloma disease activity

Monitoring disease activity is most commonly performed on peripheral blood serum and bone marrow (see Table 1).

Routine M-protein detection in blood or urine

Serum or urine protein electrophoresis (SPEP, UPEP) is used in routine clinical practice as a tool to support the diagnostic evaluation and disease monitoring of patients with MM. As SPEP/UPEP separates different types of proteins found in serum or urine, it is also suitable to identify the presence of a unique M-protein. The two methods for SPEP/UPEP are gel-based electrophoresis and capillary electrophoresis. While gel-based electrophoresis performs separation in an agarose gel, capillary electrophoresis performs separation in a small-diameter fused silica capillary. Both electrophoretic methods are robust, semi-automated, and able to monitor M-protein concentrations with similar performance in terms of sensitivity, specificity, and reproducibility [19,20]. Regardless of which method is used, as a follow-up step, immunofixation electrophoresis or immunosubtraction capillary electrophoresis is used to further characterize

Table 1. Overview of methods for disease monitoring in multiple myeloma patients.

Method	Sample	Sensitivity	Applicability	Cost	Advantages	Limitations
SPEP	Serum	~2 g/L	100%	€	Cost-effective, high throughput, robust	Poor sensitivity, M-protein half-life, t-mAb interference
IFE	Serum	~0.5 g/L	100%	€€	Cost-effective, M-protein isotype	Not quantitative, poor sensitivity, M-protein half-life, t-mAb interference
FLC	Serum	~0.001 g/L (polyclonal)	100%	€€	High throughput, sensitive for FLC	Poor sensitivity for intact M-protein, inter-institute differences
Intact protein MS	Serum	~0.1 g/L	100%	€€€	High throughput	Intermediate sensitivity, M-protein half-life
Bottom-up MS	Serum	~0.001 g/L	100%	€€€€	High sensitivity and specificity	Requires baseline sequence data and high-end MS expertise, M-protein half-life, currently RUO
NGF	Bone marrow	1 in $\geq 10^5$	100%	€€€	High sensitivity, accepted as MRD-method	Invasive, fresh sample is needed, possible sampling bias
NGS	Bone marrow	1 in $\geq 10^6$	90%	€€€€€	High sensitivity, accepted as MRD-method	Invasive, baseline sample is needed, possible sampling bias, limited global availability

IFE: immunofixation electrophoresis; FLC: free light chains; MS, mass spectrometry; RUO: research use only; SPEP: serum protein electrophoresis; t-mAB: therapeutic monoclonal antibody.

the M-protein isotype. These electrophoretic techniques are complemented by immunoassays to quantify circulating free light chains to diagnose, prognosticate and monitor patients with MM [21,22]. Electrophoretic M-protein diagnostics combined with serum FLC analyses provide robust, reliable, and high-throughput assays to monitor myeloma disease activity [23]. These methods are, however, not suitable to measure low M-protein concentrations because the polyclonal immunoglobulin background negatively impacts the sensitivity of these assays [24]. Recognition of the limited sensitivity to accurately measure low-concentration monoclonal abnormalities is reflected in the IMWG guidelines that requires "measurable" disease to meet at least one of the following three criteria: serum M-protein ≥10 g/L, urine M-protein ≥200 mg/24 h, or serum involved FLC ≥100 mg/L, provided that the FLC ratio is abnormal (involved FLC, FLCs from the monoclonal immunoglobulin are involved while FLCs from the polyclonal background are uninvolved) [3,25]. Optimized MM treatment strategies and the introduction of novel treatment modalities such as immunomodulatory drugs, proteasome inhibitors, and CD38-targeting antibodies, and more recently bispecific antibodies and CAR-T cells have greatly improved the rates and depth of responses [26–28]. For more sensitive detection of myeloma disease activity, bone marrow analysis is required.

Minimal residual disease evaluation

Bone marrow-based MRD methods allow fast examination of millions of cells (or the corresponding amount of DNA) and provide a quantitative assessment of residual myeloma cells in the bone marrow [29]. The IMWG defines MRD negativity as the absence of clonal plasma cells in the bone marrow aspirate, measured with techniques that have a minimum sensitivity to detect 1 myeloma cell in 10^5 nucleated cells [3]. The techniques that are most suitable for MRD testing are NGS or NGF, as they can reach the minimum sensitivity level required. With methodological improvements, an even higher sensitivity of 1 in 10^6 nucleated cells can be achieved with NGS [30], and NGF has a sensitivity that ranges between 1 in 10^5 and 1 in 10^6 nucleated cells. For both assays, the availability of sufficient bone marrow cells is the limiting factor [31]. Because of the complexity and challenges in harmonizing NGF, the measurements in clinical trials are often centralized in an academic laboratory. The ClonoSeq assay (Adaptive Biotechnologies Seattle, WA, USA) is currently the only Food and Drug Administration (FDA)-approved NGS assay. Along with recent advances in NGS and NGF methodologies, interest in the detection of circulating tumor cells (CTC) using NGS and NGF has recently increased. Several groups have demonstrated that CTCs are detectable at diagnosis in the majority of MM patients. Moreover, high levels of CTCs are associated with aggressive MM and correlate with a significantly worse PFS compared to low CTC levels [32–34]. However, due to the low percentage of CTCs, especially in patients undergoing treatment, only a few patients have measurable CTC levels, suggesting that CTC monitoring is currently not sensitive enough for blood-based MRD evaluation [34,35].

Imaging techniques to support evaluation of disease activity

As MM is often a patchy disease and myeloma cells may grow outside the bone marrow, there is a chance

of false negative bone marrow-based MRD test results due to sampling bias [36]. Therefore, the IMWG incorporated imaging in addition to bone marrow evaluation to better characterize MRD [3]. Magnetic resonance imaging is a sensitive, noninvasive imaging technique to detect bone involvement and to provide information on soft tissue disease and the pattern of myeloma growth in the bone marrow [37]. Positron emission tomography (PET) imaging is used to assess the metabolic activity of tumor cells [38]. A widely used glucose analogue, 2-[(18)F]fluoro-2-deoxy-D-glucose (FDG), is used to measure the uptake of glucose by tumor tissue [39]. The combination of PET with computed tomography (CT) has become a powerful imaging technique to evaluate tumor activity not only at MM diagnosis but also during therapy, and it is the current standard of care to evaluate post-therapeutic residual infiltration [40,41]. According to the IMWG criteria, imaging MRD negativity is defined when every area of increased tracer uptake at baseline on PET/CT scans has disappeared, or the uptake value of FDG has decreased to either less than the maximum standardized uptake value of the mediastinal blood pool or to less than that of the surrounding normal tissue [3]. These criteria are applicable only when MRD negativity has already been defined by NGS or NGF. Regarding the normalization of lesions after effective treatment, the prognostic and predictive value of FDG-PET/CT is superior to magnetic resonance imaging because the latter lesions can remain positive for months [42]. Limitations of FDG-PET/CT are radiation exposure, false negativity at baseline that may be due to low expression of hexokinase-2 [43], false negative results in case of hyperglycemia, and the lack of standardized criteria for evaluation of disease activity [37]. MRD status is becoming an increasingly important response parameter in randomized clinical trials, and recent studies even support the use of MRD status as a surrogate endpoint for PFS [44]. Despite the enormous gain in sensitivity and the impact of bone marrow-based MRD evaluation on the management of patients with MM, some factors limit the application of these techniques. The bone marrow aspirate is an invasive procedure that causes patient discomfort and limits repeated MRD testing. Sampling bias may occur due to hemodilution during the aspiration process and inter-operator variability [45]. However, sampling bias can be minimized by using the first pull of aspirate [46] and an optimized protocol for bone marrow sampling [47]. Bone marrow infiltration in MM is frequently heterogeneous and characterized by myeloma hotspots alternating with regions with relatively few myeloma cells, which introduces the risk of non-representative sampling [48]. Additionally, an outgrowth of extramedullary myeloma may give false negative results even after repeated bone marrow sampling. To circumvent these limitations, several groups have developed more sensitive blood-based assays to measure disease activity as potential patient-friendly future alternatives to bone marrow-based MRD evaluation.

Blood-based M-protein monitoring using mass spectrometric methods

Over the last decade, substantial progress has been made in the development of ultra-sensitive methods to detect M-protein in peripheral blood serum using MS. Both the clonotypic peptide approach (bottom-up, MS-MRD) and the intact protein approach (intact protein MS) make use of the unique mass and sequence of the M-protein to measure its concentration. Each method offers specific advantages such as high sensitivity or high throughput (Figure 1).

M-protein detection using intact immunoglobulin methods (intact protein MS)

Electrophoretic methods are widely employed for robust quantification and isotyping of M-proteins. With increasing clinical demands to detect low concentrations of M-protein, to overcome interferences from novel therapeutic monoclonal antibodies, and to shorten turn-around times, electrophoretic methods have reached their analytical limits. Mass spectrometric assays that determine the presence of monoclonal immunoglobulins by detecting intact immunoglobulin chains, as shown in Table 2, provide time-saving and accurate methods for M-protein detection. These intact protein MS methods are interpreted in a similar fashion to SPEP and immunofixation electrophoresis because monoclonal immunoglobulins present as a peak in the mass/charge ratio (m/z) mass distribution [49]. Hence, this method requires the M-protein to be more abundant compared to the polyclonal immunoglobulin background. Intact protein MS assays are highly suitable for routine diagnostics because of their rapid turn-around time and their high specificity, as was demonstrated by Murray et al. [50]. The fast run-time does, however, come at the expense of lower assay sensitivity. In 2014, Barnidge et al. from the Mayo Clinic published both an intact protein MS [51] and a bottom-up [52] method for M-protein monitoring in MM patients using the same liquid chromatography mass spectrometry (LC-MS) system. Using a 15-min gradient for both methods, the bottom-up method reached a lower limit of detection

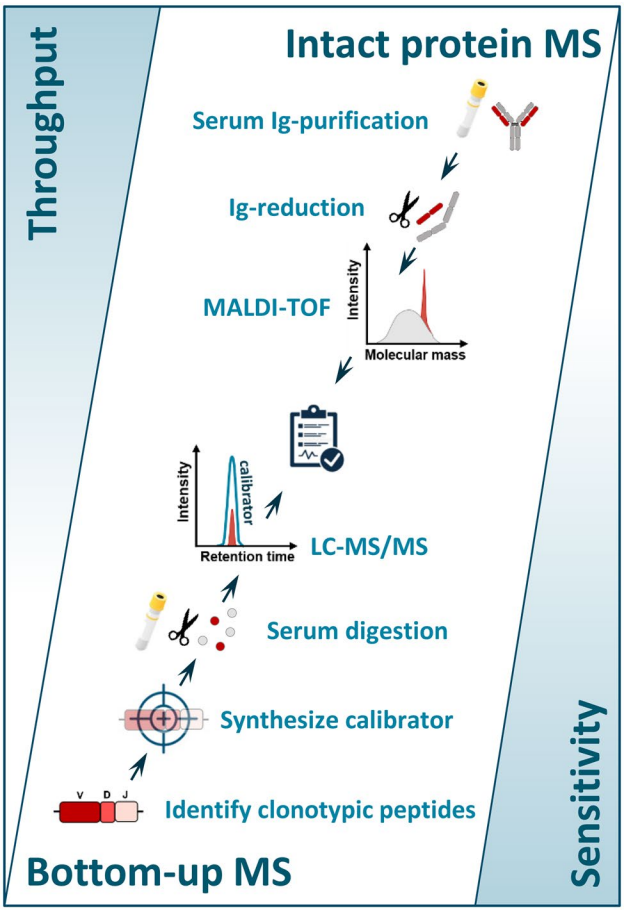

Figure 1. Graphical illustration of Intact protein MS versus Bottom-up MS. Intact protein MS measures light chain m/z using MALDI-TOF which can reach high throughput and is easier to standardize. Personalized targeting of monoclonal peptides using bottom-up MS is technically more complex but increases the sensitivity of the assay.

Table 2. Overview of intact protein MS methods to detect M-proteins.

Analytes	Immunoglobulin purification method	Measurement time per sample (min)	Instrument/Method	LLOD (mg/L)	Reference
Heavy and light chains	IgG purification (melon gel)	15	LC-ESI-Q-TOF	50 (LC) 250 (HC)	[51]
Heavy and light chains	IgG purification (melon gel) kappa and lambda purification (capture select)	24	LC-ESI-Q-TOF/ MiRAMM	5	[53]
Light chains	IgG, IgA, IgM purification Kappa and lambda purification (capture select)	<1	MALDI-TOF/ MASS-FIX	450	[55]
Light chains	IgG purification (melon gel)	35	LC-FT-ICR	30	[81]
Heavy and light chains	Kappa and lambda purification (capture select)	11	LC-ESI-Q-TOF	10	[82]
Heavy and light chains	IgG, IgA, IgM purification Kappa and lambda total purification Kappa and lambda FLC purification (EXENT-MS)	<1	MALDI-TOF/ EXENT-MS	Not reported	[56]

LC-ESI-Q-TOF: liquid chromatography electrospray ionization quadrupole time-of-flight; LC-FT-ICR: liquid chromatography Fourier transform ion cyclotron resonance.

(LLOD) of 43 mg/L and the intact protein MS method reached 250 mg/L for detecting the M-protein heavy chain. By increasing the separation time, this method, termed monoclonal immunoglobulin rapid accurate molecular mass, improved the LLOD by almost 10 times, to 5 mg/L [53]. The throughput of intact protein MS M-protein monitoring was even further accelerated by replacing the liquid chromatography-electrospray

ionization quadrupole time-of-flight (LC-ESI-Q-TOF) MS system with matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) [54–56]. MALDI-TOF technology for M-protein detection uses reduced serum samples that are spotted on a target plate embedded in a matrix [53]. The sample spots and matrix are ionized using a laser beam. The resulting charged ions are accelerated under a fixed potential that causes the ions to travel through the flight tube to the detector. Based on mass and charge, ions have a distinguishable time of flight that enables protein identification [57]. MASS-FIX, a combination of MALDI-TOF-MS and immunoglobulin enrichment into five separate Ig-fractions (IgG, IgA, IgM, kappa, and lambda) showed improved sensitivity over both SPEP and immunofixation electrophoresis with an ultra-fast run time of less than one minute for each patient and the potential to assess the M-protein isotype. Furthermore, Mellors et al. demonstrated the detection of N-glycosylation of monoclonal light chain using MASS-FIX analysis [58], which was later confirmed in a large cohort [59]. This important post-translational modification is a potential new risk marker as patients with M-protein light-chain glycosylation had an increased risk to develop clinically relevant plasma cell disorders, including amyloid light-chain (AL) amyloidosis [60,61]. Patients with IgM light chain glycosylation were at increased risk of developing cold agglutinin disease [62,63]. To also measure monoclonal free light chain (FLC), the MASS-FIX method was extended with magnetic beads that bound FLC kappa and FLC lambda to purify a total of seven Ig-fractions from serum samples [64]. This latter method has been further developed by Binding Site (Birmingham, UK) and named "EXENT-MS" [56]. Intact protein MS methods for detecting M-protein are cost-effective, high-throughput, and versatile assays for screening and monitoring [65]. However, monitoring MRD requires higher sensitivity and the measurement of clonotypic peptides rather than intact immunoglobulins.

M-protein quantification using clonotypic peptide methods (bottom-up MS)

MS targeting of clonotypic peptides originating from the variable region of the M-protein provides an ultra-sensitive patient- and disease-specific assay. In recent years, significant advances have been made within the field of M-protein detection using liquid chromatography tandem MS (LC-MS/MS) or targeted MS assays as listed in Table 3. Barnidge et al. were among the first groups to report on such an assay [51,52]. Others made improvements in sensitivity by using more advanced targeted MS techniques such as parallel reaction monitoring (PRM) instead of selected reaction monitoring (SRM) and by developing different methods for immunoglobulin purification. The term, MS-MRD, was coined for this blood-based assay as clonotypic peptide targeting allows monitoring of disease activity with a sensitivity that is similar to MRD evaluation performed on bone marrow [66]. MS-MRD assays generally include the following steps: (1) clonotypic peptide selection (targeted/personalized assay development), (2) purification of immunoglobulins in peripheral blood serum, (3) sample digestion to generate immunoglobulin peptides, (4) injection into the LC-MS system and (5) data analysis including quantification of the M-protein.

Peptide selection and targeted (personalized) assay development

Target peptides from the variable region of the M-protein can be selected from either genetic material from the myeloma cell or from *de novo* sequencing of the serum M-protein. The advantage of direct sequencing of myeloma cells is that it provides high sequence certainty of the clonotype [66]. The advantage of *de novo* M-protein sequencing is that MS-MRD becomes completely independent of bone marrow sampling [67]. RNA originating from bone marrow can be analyzed with RNAseq or amplified using a multiplex PCR reaction, and such data can be used to derive the peptide sequence from the most abundant clone, as demonstrated by Barnidge et al. [52]. This provides high certainty of the M-protein sequence. However, this method does require an invasive bone marrow aspirate, and retrieving the M-protein sequence is time-consuming. In patients with low bone marrow disease burden and lack of a dominant clone, mRNA sequencing may not provide conclusive results. Blood-based options are available for clonotypic peptide selection; however, these methods also require a minimum M-protein concentration. The first reported blood-based method to select M-protein-specific peptides is called *de novo* feature selection. In this method, a shotgun analysis is performed on a tryptic digest, followed by a *de novo* search [68]. The most important advantage of this method is the complete independence from bone marrow sampling. However, the level of sequence certainty when using single enzyme digestion is lower compared to mRNA sequencing methods. This is potentially problematic when stable isotope labeled (SIL) peptides are used for quantification because the synthesis of SIL peptides requires

Table 3. Overview of bottom-up MS methods to detect M-proteins.

Analyte	Analyte selection	LC-MS/MS method	Quantification method	Immunoglobulin purification method	Instrument	LLOD (mg/L)	Citation
Heavy chain variable region	RNA seq.	SRM	Relative signal intensity	IgG purification (melon gel)	LC-ESI-Q-TOF	43	[52]
Light chain variable region	RNA seq.	SRM	SIL peptides	–	TQS (QQQ)	15	[79]
Light chain variable region	*De novo*, Trypsin only	DDA (untargeted, MS/MS of top 15 precursors)	Relative signal intensity	Kappa or lambda purification (capture select), followed by LC purification (SDS-PAGE gel)	Q exactive – Orbitrap	1	[68]
Heavy chain variable region	RNA seq.	PRM	SIL peptides	IgG purification (melon gel)	Fusion – Orbitrap	0.2	[72,83]
Light chain and heavy chain variable region	RNA seq.	PRM	Relative signal intensity	Kappa or lambda purification (capture select)	Q exactive plus	0.1–1.5	[73]
Light or heavy chain variable region	*De novo*, multi-enzyme	PRM	SIL peptides	IgG purification (melon gel)	Q exactive	7	[71]
Light or heavy chain variable region	*De novo*, multi-enzyme	PRM	Relative signal intensity (% residual M-protein)	IgG purification (melon gel)	Orbitrap Fusion Tribrid – Q Exactive Hybrid Quadrupole Orbitrap	0.25	[70]

accurate and complete information on the peptide sequence. To tackle this problem, multi-enzyme digestion may be used to provide significantly higher sequence coverage and, because of data from overlapping peptide fragments, more accurate sequence information [69–71]. However, this method is time-consuming and labor-intensive because multiple sample digests per patient are required, and there are increased demands on reagents, mass spectrometer runtime, and data integration. Although all clonotypic peptides are derived from the variable region of the M-protein, uniqueness still needs to be assessed to exclude peptides that are identical to commonly expressed germline sequences for the variable region or are present in other serum proteins. Optimal MS-MRD sensitivity can be reached only when selected peptides trace back exclusively to the clonal sequence of the myeloma cells. Alignment with germline variable region sequences in databases such as IMGT can be used to select unique peptides [72]. Computational methods in which potential clonotypic peptides are compared to open-access databases containing immune repertoire data can be used for the automated selection of clonotypic peptides. As a quality control measure to ensure myeloma specificity, potential clonotypic peptides can be targeted in healthy control sera and can be excluded from patient monitoring when they are present in such controls. Although Langerhorst et al. showed 100% stability of clonotypic peptides during disease progression in 36 MM patients [66], clonal evolution of the M-protein sequences, a process in which the sequence of the M-protein changes during disease progression, could theoretically occur. To assess this phenomenon in patient follow-up data, at least two clonotypic peptides should be selected, preferably from both the heavy and the light chain. Monitoring at least two clonotypic peptides and internal quality control in one multiplexed MS-run provides resilience against rare clonal evolution and allows monitoring of free light chain escape.

Immunoglobulin purification

Purification of immunoglobulins in peripheral blood serum leads to a higher concentration of M-protein in these samples; this reduces the level of abundant serum proteins and increases the signal-to-noise ratio. To further improve the sensitivity of MS-MRD, several options for immunoglobulin purification are available. Zajec et al. have shown up to a 17-fold increase in LLOD in purified samples using melon gel compared to non-purified digests [72]. Melon gel provides an

easy-to-use, negative selection for IgG and IgA. It is available in a commercial purification kit (Thermo Fisher Scientific, Waltham, MA, USA) in which the resin binds all serum proteins except for IgG and IgA. Martins et al. [73]. achieved the same levels of sensitivity as Zajec et al. by using capture select resin (Thermo Fisher Scientific) to purify the immunoglobulins in peripheral blood serum. Capture-select purification methods are based on a positive selection of beads that bind either kappa or lambda, depending on the resin that is used. Bergen et al. also performed an enrichment for kappa or lambda, depending on the isotype of the M-protein, using capture select [68]. Samples were reduced using DL-dithiothreitol followed by separation using sodium dodecyl sulfate polyacrylamide gel electrophoresis, after which they selected the fragment of interest. Although this method provided a very pure immunoglobulin product, it was time-consuming. This makes it difficult to implement in routine diagnostics where fast, robust, and cost-effective methods are required.

Sample preparation and LC-MS/MS

Sample preparation steps before MS acquisition include sample reduction to break disulfide bonds connecting heavy and light chains, alkylation to prevent the re-establishment of disulfide bonds, and digestion of the serum sample. The most commonly-used digestion enzyme is trypsin, because of its robustness, efficiency, and specificity for basic residues; it generates peptides with higher charge states upon ionization [74]. Peptides in the digested samples are separated by the LC system based on hydrophobicity. The peptides are gradually injected into the MS system, they are ionized, creating charged ions, and they enter the quadrupole where specific peptides of interest are selected and fragmented. In the detector, specific fragment masses are analyzed [75]. In MS-MRD studies, different mass analyzers have been used (Table 3). A triple quadrupole mass analyzer specifically detects ion fragments of interest based on the trajectory stability of the ion in the third quadrupole [76]. Orbitrap systems trap ions in an electrostatic field in which a central electrode kept at high voltage causes the ions to move around the central electrode. The frequency of this motion is recorded and subjected to a Fourier formation. Because the frequency is proportional to the mass-to-charge ratio, ion fragments can be identified [77]. Targeted MS was established with the development of SRM and was introduced in the proteomics field about a decade ago [78]. In SRM, fragment ions are generated from precursor peptides. A pair of precursor and associated fragment ions that occur after the dissociation of the precursor are referred to as transitions. For reliable identification and quantification of a peptide, several of these fragment ions, preferably with high signal intensity, should be monitored, making the development of an SRM method a time-consuming process. A triple quadrupole mass spectrometer operated in SRM mode delivers good sensitivity and dynamic range, although the selectivity can be limited due to its low-resolution analyzers. In the first published MS-MRD assays, SRM was the only option for targeted monitoring [52,79]. When the resolution and accuracy of mass spectrometers improved, PRM emerged as an alternative to SRM. In PRM mode, a mass spectrometer records all fragment ions for a selected precursor, increasing the specificity and providing a more flexible workflow compared to SRM. As shown in Table 3, all recent MS-MRD publications used PRM to target clonotypic peptides. Another development within the field of fast high-resolution MS entails data-independent analysis (DIA). DIA is a next-generation proteomic methodology that records full MS/MS spectra of all precursors present in a sample, providing an even more flexible method [80]. Retrospective analysis is possible and allows a retrospective switch to other targets, which is not possible with SRM or PRM. DIA should provide a time-saving solution for MS-MRD because the development of a personalized PRM method for each new patient is circumvented. However, even when DIA runs are performed on high-speed/resolution MS instruments, it is anticipated that full spectra recording will compromise performance in terms of speed, sensitivity or selectivity.

M-protein quantification

For accurate monitoring of therapy response, absolute M-protein quantification is crucial. MS-MRD quantification requires a calibrator to be added to the sample before data acquisition. SIL peptides are widely regarded as the optimal internal standard for the absolute quantification of protein using LC-MS/MS. Also in the setting of MS-MRD, SIL peptides offer the best possible reference for clonotypic peptides [71,72,79]. SIL peptides are heavy labeled, synthesized copies of the clonotypic peptide with a known concentration that are spiked into the patient serum to quantitate the MS-MRD signal intensity. The disadvantage of using SIL peptides for quantification is the requirement for the synthesis of a unique standard for each patient. This is expensive and time-consuming, and it does not facilitate a standardized workflow. Using a universal calibrator to quantitate M-protein concentrations for all patients would make MS-MRD more suitable for

implementation in routine clinical diagnostics. Several options are available to relate unknown follow-up samples to, for example, SPEP-positive samples with a known concentration of the M-protein, providing a path to quantitation. Different possibilities for the use of universal calibrators for MS-MRD quantification are discussed below.

Challenges facing implementation of MS-MRD in routine clinical practice

Using MS-MRD, M-protein concentrations can be detected with a 1000-fold increased sensitivity compared to serum electrophoretic methods. The first studies that report on direct head-to-head comparisons of blood-based MS-MRD versus bone marrow-based MRD evaluation suggested that both methods had similar sensitivity to detect disease activity with similar prognostic value [18,70,73]. Whilst these encouraging results demonstrated that in the future, MS-MRD might be a less invasive alternative for MRD evaluation based on bone marrow, some challenges related to the complexity of this personalized assay lie ahead before MS-MRD methods can be implemented in routine clinical practice. These include intrinsic challenges (assay labor intensity and the use of a patient-specific calibrator), regulatory challenges (FDA/EMA clearance, assay validation and standardization), and disease-related challenges (M-protein half-life).

Intrinsic challenges

MS-MRD is time-consuming and labor-intensive

High sample throughput is an important requirement for the implementation of MS M-protein analysis in routine diagnostics. In 2018, Kohlhagen et al. provided a best practice example of the automation of their MASS-FIX method (including pre- and post-analysis) for implementation in their routine M-protein diagnostics workflow [84]. To date, all described MS-MRD assays have been based on PRM methods using personalized internal standards and rely on manual data analysis. To improve sample throughput, several elements of the method need automation. Automated liquid handling would improve workflow and precision. Furthermore, the workflow should include automated clonotypic peptide selection as this crucial part of the assay is time-consuming and requires expertise. Important criteria for peptide selection include the uniqueness of clonotypic peptides originating from the immunoglobulin variable region and raw signal intensity [68,71,73]. Furthermore, the algorithm for selecting clonotypic peptides should include criteria based on linearity and sensitivity in a dilution series and should meet quality control aspects such as the absence of targeted signal in healthy control samples. Another time-consuming bottleneck is the manual analysis of MS-MRD data. By logging the patient intake data and selected clonotypic peptides for each patient, data analysis of follow-up samples could be automated as well. MS instrument operation still requires expertise, and simplification of instrument operation is needed for broad clinical implementation of MS-MRD and for use in laboratories with less MS experience [85]. Recent developments within the field of LC-MS/MS assays, such as DIA, could obviate the need to develop a personalized PRM method for each new patient. To date, no MS-MRD application using DIA has been published. Although DIA has the potential to be part of a highly automated "one MS-MRD assay fits all", it does require a high-speed and high-resolution mass spectrometer. Compared to PRM, DIA will generate even more complex data as it records full MS/MS spectra that further challenge the automation of the data analysis.

Patient-specific calibrator

SIL peptides offer, besides a calibrator to quantify MS-MRD data, the best possible reference for clonotypic peptides in terms of retention time, ion mobility, and fragmentation spectra. Unfortunately, the use of SIL peptides is expensive and time-consuming because each patient requires the synthesis of unique SIL peptides. Furthermore, using SIL peptides requires a high certainty of the M-protein peptide sequence. To overcome this problem, a universal, off-the-shelf calibrator could be used to replace SIL peptides. A stable isotope labeled universal monoclonal antibody (SILuMAb, Sigma Aldrich, Saint Louis, MO, USA) was described by Schokker et al. to function as an internal standard in the measurement of trastuzumab and pertuzumab; one SILuMAb peptide selected as a calibrator peptide displayed excellent assay linearity over 3 log scales [86]. This method could be applied to MS-MRD as well by comparing ratios of SILuMAb to the clonotypic peptide. In this way, multiple peptides per patient could be assessed in the quantification of the M-protein without the need for ordering new SIL peptides. Zajec et al. explored an alternative workflow for M-protein quantification using tandem mass tag labeling in which two samples with different M-protein concentrations were labeled with different tandem mass tags before combining them. The M-protein concentration of one

sample was known and was used to recalibrate the second sample that had an unknown M-protein quantification [83]. Such labeling methods indeed circumvent the need to use unique labeled peptides, but the process of labeling the samples with tandem mass tag reagents requires an extra sample preparation step. Although the methods presented by both Zajec et al. and Schokker et al. require at least one sample with a known M-protein concentration, usually assessed by SPEP, the potential of using a universal calibrator to simplify MS-MRD is demonstrated. For some patients, however, such samples are not available, and it is possible to report the response to therapy only in terms of the percentage change of M-protein concentrations over time [73,87].

Regulatory challenges

Analytical and clinical validation

Worldwide, several groups have invested heavily in improving the performance of MS-MRD. Thus there is no consensus method at present for the performance of MS-MRD, and studies performed to date are on relatively small cohorts (Table 3). Analytical validation of a mature MS-MRD method should include an assessment of its sensitivity, specificity, accuracy, turn-around times, reproducibility, and linearity. Prior to approving MS-MRD in a routine clinical setting, extensive clinical validation on large retrospective and prospective cohorts of MM patients should be performed. Clinical validation should entail MS-MRD feasibility, its prognostic value, its capacity to monitor response to therapy, and its clinical concordance with bone-marrow-based MRD evaluation. The goal of these studies is to assess the complementary clinical value of MS-MRD but also its potential shortcomings. Furthermore, they will provide information about sampling time points and frequencies in the assessment of a patient's MRD status. Whilst MS-MRD provides a less invasive method to assess MRD, unnecessary sampling should still be avoided. In a more mature development stage, clinical validation of MS-MRD should be performed in prospective MM cohorts to shadow other MRD detection methods or study how MS-MRD can be implemented to facilitate MRD-guided therapy.

FDA/EMA clearance

Diagnostics tests performed in sites other than where they were developed require FDA (USA) or EMA (Europe) clearance. In 2020, the FDA published regulatory considerations for MRD products as guidance on the requirements for approving an *in vitro* diagnostic device to measure MRD as well as the use of MRD as a biomarker in clinical practice [88]. In their non-binding recommendations, the FDA is neutral regarding the technology platform that should be used to assess MRD. However, the FDA does state that the assay procedure, reagents, and analysis are to be prespecified [88]. Furthermore, the complete assay, from sample collection to system output, should be validated analytically for the intended use using relevant clinical samples. Regarding the applicability of MRD status in clinical practice, the FDA differentiates various types of biomarkers: diagnostic, prognostic, predictive, efficacy response, and monitoring. Understanding which biomarker attributes apply to its proposed use is important when validating the MRD status for that specific use [88]. The significant improvements in clinical outcomes of MM have spurred interest in the use of MRD status as a potential surrogate endpoint for survival to expedite drug development. Regarding its use as a surrogate endpoint for PFS, the FDA states: "the strength of evidence to support surrogacy depends on (1) biological plausibility of the relationship, (2) demonstration in epidemiological studies of the prognostic value of the surrogate endpoint for the clinical outcome, and (3) evidence from clinical trials that treatment effects on the surrogate endpoint correspond to effects on the clinical outcome." As the M-protein is a thoroughly studied biomarker and is used in routine diagnostics, the biological plausibility of its relationship with disease activity and its prognostic value are widely accepted. Furthermore, in a first proof of concept study with 41 patients from the IFM 2009 trial, the MS-MRD data of Langerhorst et al. indicated that MS-MRD negative blood-tests correlated with a longer PFS [18]. However, the prognostic value of MS-MRD negativity as well as the relationship between treatment outcome and MS-MRD status should be validated in large, independent cohorts of MM patients. Also, crucial information regarding the MS-MRD threshold can be gathered. Additionally, when MS-MRD is used as a biomarker to guide therapy decisions, it is important to consider that the sensitivity of the assay should be at least 10-fold below the decision-making threshold. Similarly, in the current guidelines, MRD negativity is defined as detection of less than one in 10^5 cells; thus the assay should be optimized and validated to have an analytical sensitivity of at least one in 10^6 cells.

Standardization and harmonization

To ensure reliable test results and minimize the risk of incorrect interpretations, harmonization among laboratories that measure the same measurand should be established [89,90]. The International Organization for

Standardization (ISO) has released requirements for the implementation of harmonization protocols (ISO 21151:2020) in cases that lack certified reference materials [91]. This applies to MS-MRD because no certified reference material is available for the unique patient measurand. Nonetheless, harmonization can be achieved by periodically providing a panel of clinical samples that are analyzed by each individual laboratory performing MS-MRD assays, followed by result comparison for agreement. As part of a harmonization protocol, a definition of the threshold for MS-MRD negativity should be considered. In contrast to the internationally-defined threshold for bone marrow-based MRD, no such threshold for MS-MRD assessment has been determined yet. Therefore, relevant cutoff values defining a relapse should be considered. For the purpose of harmonization, a comparison between multiple centers should be performed to show the comparability between institutes. It is anticipated that current developments such as automated liquid handling and automated data analysis to improve assay robustness and turn-around times are expected to positively impact the reproducibility of MS-MRD.

Disease related challenges

M-protein half-life

The M-protein is a biomarker of myeloma disease activity. A confounding factor is the half-life of the M-protein in the blood, which causes a delay between myeloma cell elimination and a decrease in M-protein concentration. For accurate reflection of disease activity, the half-life of the M-protein isotype must be taken into account (Figure 2(A)). Different immunoglobulins have different clearance rates: the half-life of IgG is ~23 d [92], of IgA, ~6 d [93], and of free light chains, 2–6 h [94]. For IgG, this issue is even more complex because of IgG recycling by the neonatal Fc receptor that protects IgG from catabolism [92]. At high IgG concentrations, as often occurs in newly diagnosed myeloma patients with an IgG M-protein, the neonatal Fc receptor becomes saturated and strongly diminishes the IgG half-life [95]. Therapy effectiveness can be more accurately assessed if the slope of the M-protein decrease is analyzed, taking the isotype-specific half-life of the M-protein into account. Figure 2(B) illustrates that hypothetical patient 1 seems to have a better response, as indicated by the slope of the M-protein response, compared to hypothetical patient 2. However, taking into consideration that patient 2 secretes an IgG M-protein with a relatively long half-life, it can be concluded that patient 2 effectively has the best response to therapy.

Defining potential clinical applications for blood-based MS-MRD

It is anticipated that MS-MRD blood testing will provide additional information on the depth of response only in patients in whom electrophoretic and free light chain results have normalized. MS-MRD could potentially optimize patient management in various ways as shown in Figure 3. Frequent monitoring of disease activity beyond stringent complete response could reveal prognostic markers such as therapy response kinetics and relapse kinetics. Moreover, MS-MRD could play a role in reducing the number of invasive bone marrow aspirations in a patient. MS-MRD could, for example, provide information to optimize the timing of bone marrow sampling, or could help to assess

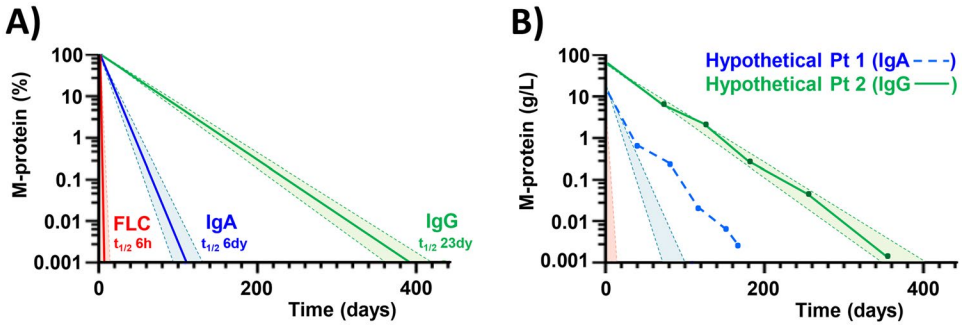

Figure 2. Schematic representation of the impact of serum half-life (t1/2) on M-protein monitoring. (A) Depending on the Ig t1/2 of the Ig-isotype, there is a certain delay between the lysis of clonal plasma cells and the decrease in serum M-protein. Responses are shown for hypothetical immediate and 100% effective therapeutic intervention in patients with an IgG-, IgA-, and FLC-M-protein. Based on the M-protein isotype and the response kinetics, therapy effectiveness can be calculated. (B) In clinical practice this would mean that hypothetical patient 2 (IgG M-protein, continuous line) experiences a 100% effective therapy response (inside IgG shaded area). Although hypothetical patient 1 (IgA M-protein, dotted line) has a steeper response curve, the therapy response is less effective (outside IgA shaded area). t1/2 = half-life.

sustained MRD negativity. On the other hand, MS-MRD may have complementary value to bone marrow-based MRD monitoring, for example, to address the unmet need of MRD monitoring in patients with extramedullary disease. Moreover, the minimally invasive character of MS-MRD allows dynamic MRD monitoring, which makes it ideally suited for early relapse detection and for the implementation of MRD-guided treatment decisions. These could include a switch in therapy, stopping maintenance therapy, or initiation of relapse treatment when MRD negativity is lost. The value of each of these potential clinical applications, however, should be investigated and evaluated in large patient cohorts.

Early relapse detection and MS-MRD guided therapy

Because of its blood-based nature, MS-MRD is well suited for frequent monitoring, which allows longitudinal analysis of disease activity. This provides more detailed information about clinical response and permits earlier detection of disease progression, as demonstrated by Noori et al. who showed that the MS-MRD blood test on average detected relapse 442 days earlier than routine blood tests [96]. Furthermore, longitudinal M-protein monitoring and early relapse detection could provide information upon which treatment decisions could be made (MS-MRD guided therapy). Figure 3 demonstrates schematically several scenarios on how dynamic MRD monitoring could provide information that might aid patient management. Accordingly, a patient presenting with increased M-protein levels directly after stopping treatment would benefit from an extended period of therapy. On the other hand, a patient presenting with stable M-protein levels could benefit from a controlled treatment-free period to improve the quality of life.

Optimize timing of bone marrow biopsies

In addition to MRD evaluation, bone marrow aspiration yields the possibility of obtaining valuable information such as the presence of specific prognostic cytogenetic

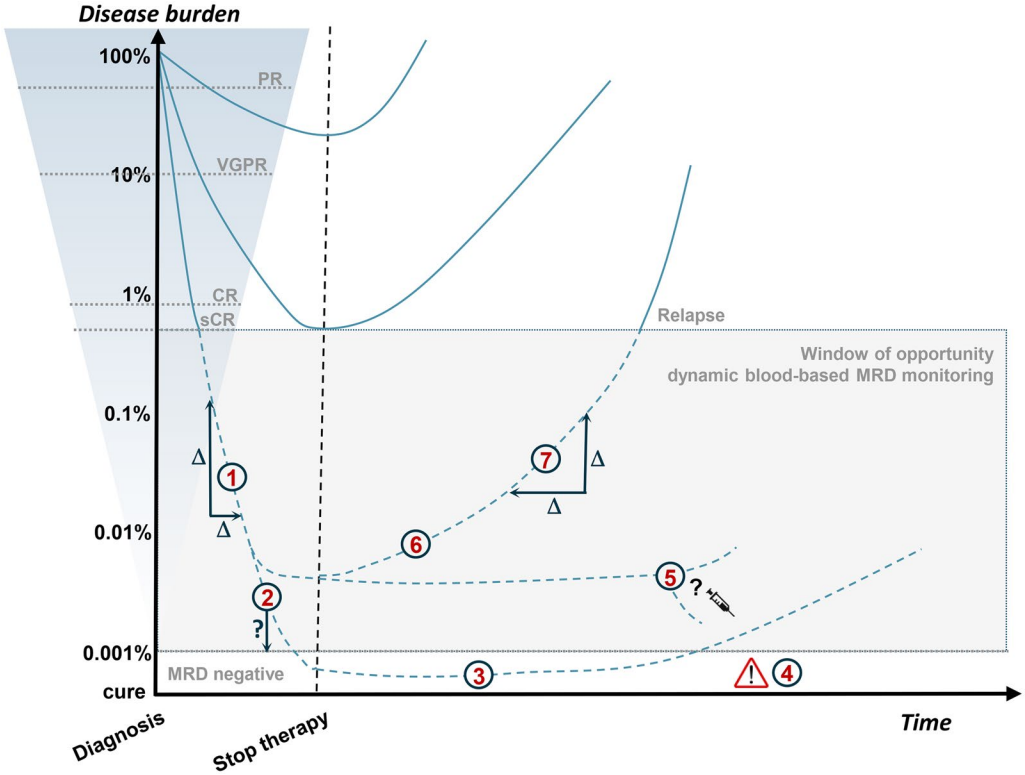

Figure 3. Schematic representation of therapeutic response evaluation. Visualization of the paradigm that deep responses are prognostic for longer survival. Current blood-based assays are not sensitive enough to monitor disease activity beyond sCR (dotted lines). The shaded box indicates the window of opportunity for more sensitive blood-based MRD monitoring with several potential clinical applications: (1) Therapy response kinetics as a potential prognostic marker; (2) Optimize timing of bone marrow sampling; (3) Diagnose sustained MRD-negativity; (4) Disease monitoring of patients with false-negative MRD results caused by extramedullary disease; (5) MRD-guided treatment decisions; (6) Early relapse detection; (7) Relapse kinetics as a potential prognostic marker. PR: partial response; VGPR: very good PR; CR: complete response; sCR: stringent complete response; MRD: minimal residual disease.

abnormalities and bone marrow immune reconstitution that cannot be obtained by MS-MRD. Furthermore, in the rare event of MM patients who do not secrete an M-protein, referred to as non-secreting MM, disease activity cannot be monitored using MS-MRD. For these reasons, we anticipate that MS will never completely replace existing MRD tests in bone marrow but will have clinical value as a companion method. Retrieving a bone marrow aspirate is an invasive procedure that is traumatic for the patient, and having to undergo this procedure multiple times during a period of intense treatment has a negative impact on the quality of life. Minimizing the biopsy frequency while still retrieving all the important information about the disease status requires good timing. MS-MRD could be used as an indicator to make informed decisions on when to perform invasive bone marrow MRD evaluation.

Therapeutic drug monitoring

Therapeutic monoclonal antibodies (t-mAbs) can cause interference in serum protein electrophoresis and immunofixation and this problem becomes increasingly difficult when a patient is treated with a cocktail of t-mAbs [97]. Because unique monoclonal peptides are monitored with MS-MRD, t-mAbs do not interfere in this assay. In the same MS-MRD analysis, it is possible to perform therapeutic drug monitoring of t-mAbs because each t-mAb has its own unique variable region carrying unique peptides [98]. Noori et al. analyzed patients treated with a combination of nivolumab and daratumumab and showed that MS-MRD could assess both M-protein concentrations and t-mAb concentrations in a single assay. These results show the potential to expand the number of serum protein analytes measured in one multiplex MS-MRD assay.

Detecting extramedullary disease

The reported incidence of extramedullary disease ranges from 0.5 to 4.8% in newly diagnosed MM patients, and from 3.4 to 14% in relapsed or refractory MM patients [99]. PET/CT or magnetic resonance imaging scans can detect extramedullary tumors, and several studies have shown that patients with extramedullary disease have a worse prognosis [100,101]. Because MS-MRD detects the M-protein in peripheral blood serum regardless of tumor location, MS-MRD has the potential to detect small tumor burden even in patients without bone marrow involvement. In rare cases, but more frequently in advanced stages of MM, involvement of the central nervous system is observed. The prognosis of patients with leptomeningeal involvement is poor, with a reported average survival of <6 months caused in part by poor penetration of anti-MM agents across the blood-brain barrier [102,103]. We have previously shown that MS-MRD can be applied to other matrices such as cerebrospinal fluid [104]. This provides the opportunity to monitor intrathecal M-protein production as a biomarker for central nervous system involvement. In the same sample, MS-MRD can also measure t-mAb concentrations as a tool to assess drug penetrance in the cerebrospinal fluid.

Conclusion

The future direction of MRD assessment in MM is toward more sensitive and less invasive technology. Ultra-sensitive detection of circulating M-protein in blood samples using LC-MS/MS now provides a dynamic overview of the patient's MRD status through a less invasive procedure that is not negatively affected by the quality of the input material, as is the case with bone marrow aspirates due to hemodilution and the patchy nature of the disease. Nonetheless, several technical aspects of MS-MRD, including cutoff values, compensation for M-protein half-life, and standardization and automation of this personalized assay, remain critical areas for further investigation. Dynamic blood-based MRD monitoring will provide more detailed information on how individual patients respond to treatment and allow early detection of disease relapse. Upcoming clinical validation studies should focus on ways to use this information in clinical practice. These studies should be performed alongside existing methodologies for MRD evaluation to investigate the complementary value of MS-MRD.

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