Mass Spectrometry for Identification, Monitoring, and Minimal Residual Disease Detection of M-Proteins

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BACKGROUND: Monoclonal gammopathies (MGs) are plasma cell disorders defined by the clonal expansion of plasma cells, resulting in the characteristic excretion of a monoclonal immunoglobulin (M-protein). M-protein detection and quantification are integral parts of the diagnosis and monitoring of MGs. Novel treatment modalities impose new challenges on the traditional electrophoretic and immunochemical methods that are routinely used for M-protein diagnostics, such as interferences from therapeutic monoclonal antibodies and the need for increased analytical sensitivity to measure minimal residual disease.

CONTENT: Mass spectrometry (MS) is ideally suited to accurate mass measurements or targeted measurement of unique clonotypic peptide fragments. Based on these features, MS-based methods allow for the analytically sensitive measurement of the patient-specific M-protein.

SUMMARY: This review provides a comprehensive overview of the MS methods that have been developed recently to detect, characterize, and quantify M-proteins. The advantages and disadvantages of using these techniques in clinical practice and the impact they will have on the management of patients with MGs are discussed.

Introduction

Monoclonal gammopathies (MGs) are plasma cell disorders defined by the clonal expansion of plasma cells, resulting in characteristic excretion of a monoclonal immunoglobulin (Ig; M-protein). MGs encompass a broad spectrum of clinical disorders ranging from asymptomatic, benign MG of undetermined significance to life-threatening diseases such as multiple myeloma (MM) and amyloid light chain (AL) amyloidosis (1).

M-protein detection and quantification are integral parts of diagnosis and monitoring of MG (2). M-protein may consist of intact monoclonal Ig and/or monoclonal fragments such as free light chains (FLC) that can be detected in serum and/or urine. M-protein diagnostics is most commonly performed using electrophoretic methods, supplemented with additional assays for quantification and clonality testing (3). Nonetheless, both traditional electrophoresis and immunochemical methods have analytical limitations that include standardization issues among different methods; poor analytical sensitivity, which hampers detection and/or accurate quantification of small M-proteins; and disease activity that remains unnoticed in patients with nonsecretory myeloma (4).

Novel treatment modalities for MM have led to deeper responses, resulting in an increased percentage of patients that obtain stringent complete response (sCR), in which residual disease can no longer be detected using routine diagnostics in blood and/or urine (5). Because many patients who obtain sCR will eventually relapse, analytically more sensitive assays capable of measuring minimal residual disease (MRD) are urgently needed. In addition, the introduction of therapeutic monoclonal antibodies (t-mAbs) can directly hamper traditional M-protein diagnostics because it can be challenging to distinguish the human(ized) t-mAbs from the endogenous M-protein.

Each M-protein is derived from recombination and somatic hypermutation events of both the heavy- and light-chain loci of the clonal B cell. Consequently, M-protein has both a unique amino acid sequence and unique molecular mass. Routine M-protein diagnostic methods, including electrophoretic and immunochemical methods, do not make use of these unique

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M-protein features, beyond the general region of electrophoretic migration. Mass spectrometry (MS) is ideally suited to making accurate mass measurements or targeted measurements of unique M-protein peptides. Therefore, it is not surprising that new MS-based methods for the detection and quantification of M-proteins appeared in the literature beginning in 2014. Some of these novel methods have already been implemented in routine diagnostics. We anticipate that, in the near future, MS will play an increasingly important role in the field of M-protein diagnostics.

In this review we provide a comprehensive overview of current MS methods that can be applied to detect, characterize, and quantify M-proteins. The advantages and disadvantages to using these techniques to complement routine M-protein diagnostics and the impact they will have on the management of patients with MG are discussed.

Routine M-Protein Diagnostics

M-protein is a serum biomarker that relates directly to the clonal plasma cell burden in a patient with MG. The secreted M-protein can be used as a screening tool for the identification of MG and as a quantitative biomarker for disease prognostication to follow the course of disease and to monitor response to therapy. M-protein diagnostics are performed using high-resolution and semiautomated electrophoretic methods that are supplemented with additional assays for quantification and clonality testing (3).

Serum protein electrophoresis (SPE) is performed using either agarose gel electrophoresis or capillary electrophoresis. These electrophoretic methods are commonly used for M-protein screening and quantification. Further characterization of the M-protein isotype is typically performed using immunofixation electrophoresis (IFE) or immunosubtraction–capillary electrophoresis. Turbidimetric and nephelometric analyses are performed to quantify total IgG, IgA, IgM, FLC, and heavy–light chain pairs (2, 3). Katzmann et al. (6) have studied which panel of serologic tests is most cost-effective to screen for MG in a large cohort of patients with various plasma cell proliferative disorders. The heterogeneity of M-proteins and the limitation of each individual assay necessitates the use of multiple tests.

Numerous international guidelines provide recommendations for M-protein diagnostics of patients with a suspected MG and for patient follow-up (3, 7–9). Despite these guidelines, test algorithms for M-protein diagnostics vary widely across laboratories (10). M-protein quantification is further challenged by the analytical limitations and interferences observed both with electrophoretic methods and immunoassays applied within the field of M-protein diagnostics (4, 11, 12). The actual
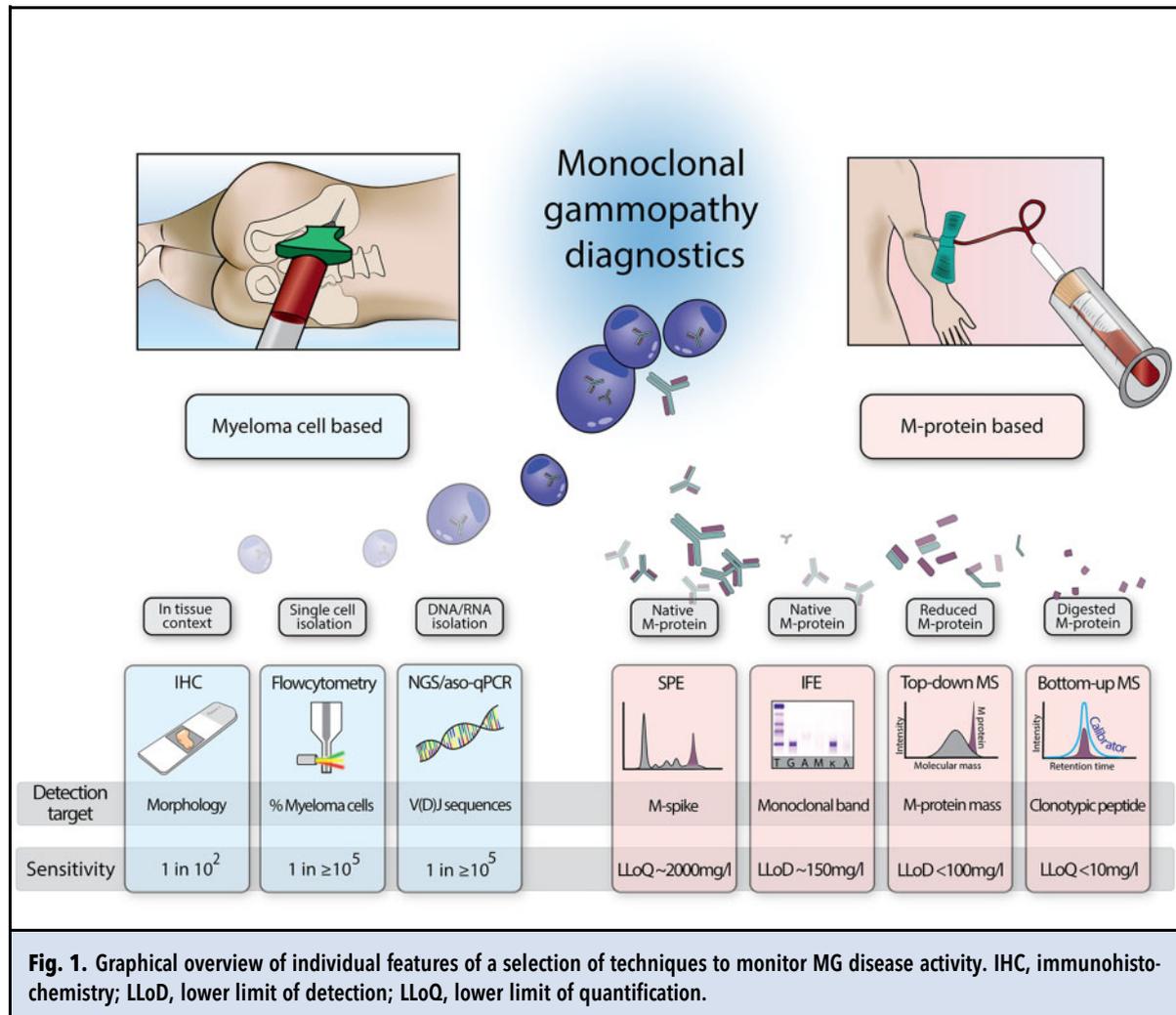
spike of the M-protein as part of electrophoretic quantification remains a subjective procedure with suboptimal quantification of small M-proteins and those that comigrate with other abundant serum proteins, for example, in the β region (4, 13). Recognition of the imprecision and inaccuracy of measurements of low-concentration monoclonal abnormalities is reflected in the International Myeloma Working Group (IMWG) guidelines that define a "measurable" M-protein as one that meets at least 1 of the following 3 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 (14).

New treatment modalities have greatly improved the rates and depth of responses in patients with MM in the past decade (15, 16). Because an increasing percentage of newly diagnosed MM patients obtain sCR, new assays need to be developed that can identify responses beyond conventionally defined sCR.

MRD Testing

Driven by the evolving framework of more effective multidrug treatment protocols, new methods have been developed to detect and quantify MRD. Current methodologies to assess MRD in MM patients focus on molecular and flow cytometric techniques performed on bone marrow aspirates (5, 17). It is evident that among patients with MM who achieve sCR, MRD assessment by multicolor flow cytometry (MFC), allele-specific oligonucleotide–quantitative PCR (ASO-qPCR), or next-generation sequencing (NGS) can play an important role in patient management. MRD status is a major prognostic factor (18). Moreover, MRD assessment can be applied to assess treatment effectiveness (19). Consequently, new IMWG consensus criteria for MRD assessment have been defined that reach beyond the detection of the present therapy response criteria (20). Generally, MRD negativity is defined by the absence of clonal plasma cells in bone marrow aspirates using methods with a minimum detection capability of 1 in ≥10^5 nucleated cells.

The cellular method (MFC) and molecular methods (ASO-qPCR and NGS) to assess MRD allow the examination of millions of bone marrow cells or the corresponding amount of DNA (Fig. 1). Each technique has advantages and disadvantages that need to be considered (Table 1). The various MRD methods and their test characteristics have been extensively reviewed elsewhere (5, 17, 19). Characteristics of an ideal MRD assay are high sensitivity, specificity, and reproducibility; feasibility for all MM patients; standardization among institutes; small sample volume; easy applicability; rapid turnaround time; and cost-effectiveness. None of the currently described methods to assess MRD meet all

Fig. 1. Graphical overview of individual features of a selection of techniques to monitor MG disease activity. IHC, immunohisto-chemistry; LLoD, lower limit of detection; LLoQ, lower limit of quantification.

ideal test requirements. To assess differences in test characteristics in individual patients, the IMWG encourages inclusion of both MFC and NGS methods in prospective trials. This also allows direct comparison between the cellular methods that measure percentage of myeloma cells and the molecular methods that measure myeloma-specific gene sequences. It is further advised that MRD assessment should not be limited to a single time point because MRD kinetics over the disease course provide more robust evaluation of disease control in patients with MM after achieving sCR (20).

The strongest limitation of the methods described is that disease monitoring must be performed on bone marrow aspirates, which introduces the risk of nonrepresentative sampling resulting from tumor heterogeneity (21). The patchy nature of the disease has a direct negative impact on the reported results of these methods,

and extramedullary MM outgrowth may give false-negative results even after repetitive bone marrow sampling. Another potential limitation is the complexity of these techniques, which makes them costly and difficult to standardize (22). In addition, the need for repetitive bone marrow punctures for patient follow-up is a physical burden that reduces the quality of life for individual patients.

Evaluation of MRD in peripheral blood would represent an attractive minimally invasive alternative to circumvent the noted disadvantages of MRD assessment in bone marrow. Studies investigating the possibility of detecting MM disease activity in peripheral blood have emerged that use MFC on circulating myeloma cells and sequencing of tumor circulating DNA. Taken together, myeloma-specific targets in peripheral blood are available for evaluation of myeloma disease activity at diagnosis (23). However, myeloma cells and tumor

Table 1. Characteristics of techniques to monitor multiple myeloma disease activity in bone marrow and serum.

	Bone marrow sampling			Serum sampling			
	MFC	ASO-qPCR	NGS	SPE/CE	IFE/IS-CE	Top-down MS	Bottom-up MS
Applicability, %	~100	~65	~90	~100	~100	~100	~100
Quantitative	Yes (clonal PCs)	Yes [V(D)J seq.]	Yes [V(D)J seq.]	Yes (M-spike)	No (visual M-peak)	No (arbitrary units)	Yes (internal standard)
Analytical sensitivity	1 in $\geq 10^5$	1 in $\geq 10^5$	1 in $\geq 10^5$	LLoQ ~2 g/L	LLoD ~150 mg/L	LLoD $\leq$100 mg/L	LLoQ $\leq$10 mg/L
Baseline sample	Important but not mandatory	Mandatory	Mandatory	Not needed	Not needed	Not needed	Important but not mandatory
Fresh sample	Needed (<36 h)	Not needed[a]	Not needed[a]	Not needed	Not needed	Not needed	Not needed
Sample volume	$\geq$5 x 10^6 cells[b]	$\geq$1 x 10^6 cells[b]	$\geq$1 x 10^6 cells[b]	500 μL	500 μL	$\leq$ 100 μL	$\leq$ 100 μL
Nonrepresentative sampling	Extramedullary and patchy disease			Non-secretory multiple myeloma (no M-protein biomarker)			
Turnaround time	2-3 h	BS: 3-4 weeks FU: $\leq$ 5 days	$\leq$10 days	$\leq$3 days	$\leq$5 days	$\leq$1 days	BS: 4-5 weeks[c] FU: ~5 days
Global availability	Intermediate	Limited	Limited	High	High	Few sites RUO[d]	Few sites RUO

BS, baseline sample (target identification); CE, capillary electrophoresis; FU, follow-up sample; IS, immunosubtraction; LLoD, lower limit of detection; LLoQ, lower limit of quantification; PC, plasma cell; RUO, research use only; M-spike, spike of the M-protein.

[a] DNA must be extracted within 36 h, analysis performed on archived DNA.
[b] More cells increase sensitivity.
[c] Without internal standard, the turnaround-time is equal to FU.
[d] Implemented in routine diagnostics at the Mayo Clinic in 2018.

circulating DNA are present at much lower levels in peripheral blood compared with the bone marrow. Consequently, disease activity measured at diagnosis becomes undetectable soon after effective therapy, even among electrophoretic-positive patients (*24*). For that reason, these methods cannot be used for early detection of disease recurrence.

In summary, MFC, ASO-qPCR, and NGS are currently available molecular methods for MRD assessment in bone marrow that provide significantly improved sensitivity compared with conventional M-protein diagnostics. All 3 methods reach detection capabilities of at least 10^{-5}, defined as the detection of 1 MM cell among a background of at least 10^5 normal leukocytes. Because of the limited sensitivity of similar methods applied on peripheral blood, MRD investigation has been restricted to bone marrow. This may change in the near future with emerging novel methods for M-protein detection using MS.

Immunoglobulin Measurements Using MS

The impact of MS on laboratory diagnostics lies in both novel biomarker discovery and improved capacity to measure clinical analytes. MS has a long history, primarily for use in small-molecule quantification applied for confirmations of drugs of abuse, newborn screening, and screening for steroid hormones (*25*).

MS methods that measure proteins were implemented much later in clinical laboratories because these assays are more complex to implement and require larger investments in terms of trained staff and equipment (*26*). Increases in the linear dynamic range, as well as improved speed, resolution, and mass measurement accuracy, have made these instruments an attractive alternative for characterizing proteins. More user friendly and more robust, newer generation MS instruments have begun to play a role in clinical diagnostics (*26*).
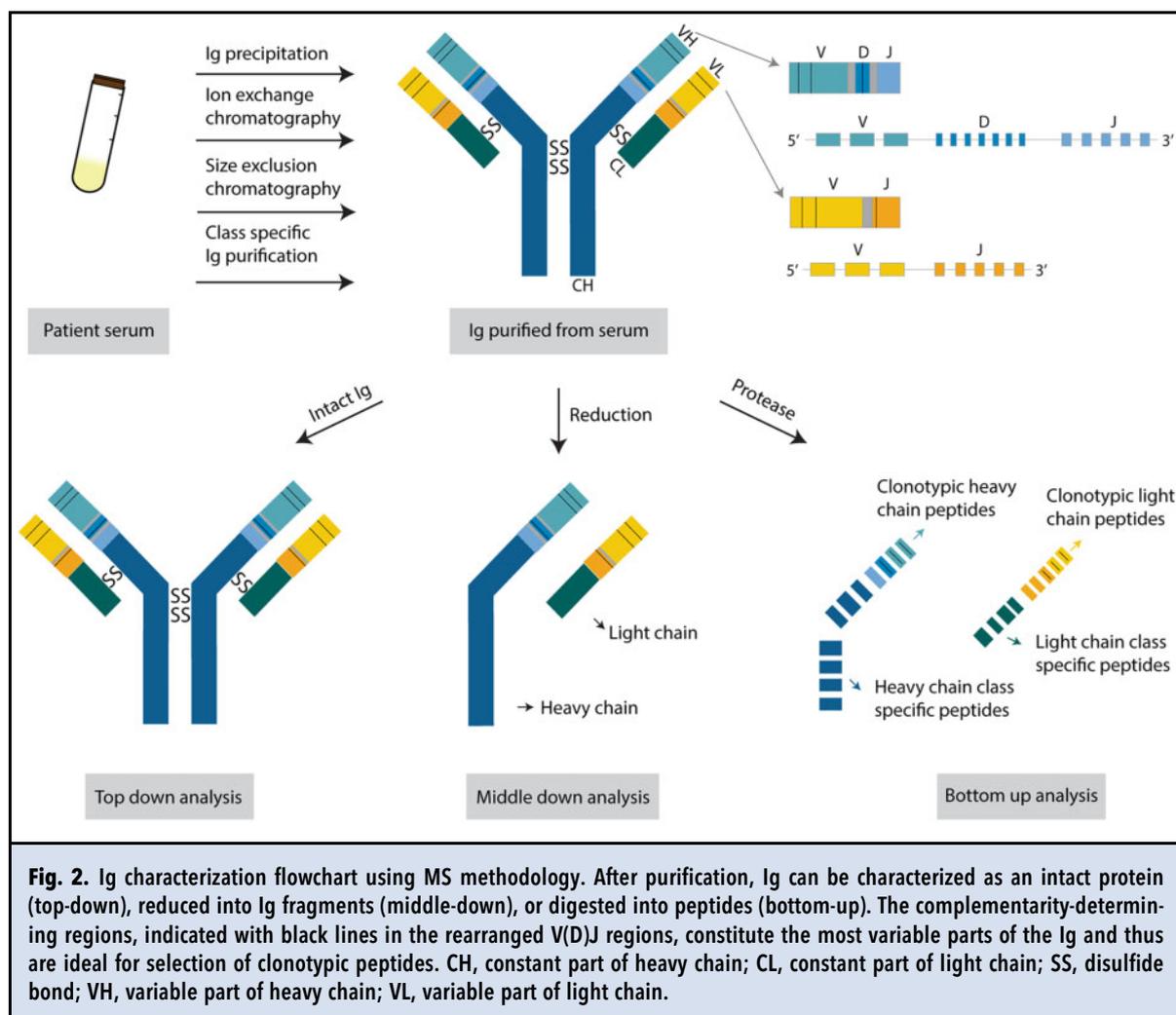
LC-MS is an analytical chemistry technique that combines the physical separation capacity of liquid chromatography with the mass analysis capacity of MS. This technique can be used to analyze complex samples. With the introduction of targeted LC-MS, quantification of protein biomarkers by measuring peptide surrogates has become feasible. As a result, different groups have pioneered methodology for Ig quantification using peptides derived from tryptic digestion of the constant Ig regions (*27*, *28*). In 2014, both groups published LC-MS/MS methods with stable isotope-labeled internal standards for quantification of total serum Ig and IgG subclasses (*27*, *28*). Our group demonstrated that accurate LC-MS/MS multiplex measurements of Ig heavy and light chains allowed complete Ig profiling including serum FLC quantification (*29*). Van de Gugten et al. (*30*) used an optimized version of the IgG subclass

LC-MS/MS method to demonstrate an apparent IgG4 cross-reactivity with immunonephelometric IgG subclass measurements. This cross-reactivity explains the discrepancies found between total IgG measurements and the total sum of the individual IgG subclasses observed in patients with IgG4-related disease. In addition to protein quantification, the rapid improvement in MS-based proteomics reveals structural Ig features that were previously unavailable with other techniques such as sequence information, polyclonal mass distributions, Ig glycosylation, and other posttranslational modifications (*31–33*).

MS as a Novel Method for M-Protein Measurements in Peripheral Blood

Based on existing literature on analysis of t-mAbs (*34*), a concept emerged that MS-based methods could be applied to measure patient-specific unique features of an M-protein. Proteomic methods are typically classified by preanalytical Ig processing into top-down, middle-down, and bottom-up (Fig. 2). The intact Ig is the starting analyte in top-down MS, and the fragmentation pattern further elucidates information on the primary structure. Conversely, bottom-up MS refers to the process in which the Ig is enzymatically digested into peptides. The Ig primary structure is inferred from the peptide sequences that are obtained by LC-MS/MS. These methods can be refined by reduction of the Ig into smaller fragments that can either be analyzed intact (middle-down) or after further digestion into peptides (middle-up) (*31*, *35*).

Important factors that contribute to optimal sensitivity and specificity of these MS methods are chemical reagents and methods used to isolate Ig and further cleave or digest these into fragments. Ig isolation decreases interference from other abundant proteins such as albumin in serum. Ig isolation can be achieved by physicochemical fractionation such as Ig precipitation, ion exchange chromatography (based on net charge), or size exclusion chromatography (based on size or molecular shape). Class-specific Ig purification can be achieved by protein A, protein G, or protein L affinity chromatography or immune capture directed against specific regions of the Ig of interest (*36*). Cleaving Ig into smaller fragments through reduction of disulfide bonds, for example, with dithiothreitol or by enzymatic Ig cleavage will result in more manageable and more specific Ig fragments for further MS characterization. Peptides produced by further enzymatic digestion of these Ig fragments provide the input material for bottom-up MS profiling. Figure 1 provides a graphical overview of the MS methods to measure serum M-protein and their complementary value to other techniques that can be used to measure disease activity in blood and bone marrow of MG patients.

Fig. 2. Ig characterization flowchart using MS methodology. After purification, Ig can be characterized as an intact protein (top-down), reduced into Ig fragments (middle-down), or digested into peptides (bottom-up). The complementarity-determining regions, indicated with black lines in the rearranged V(D)J regions, constitute the most variable parts of the Ig and thus are ideal for selection of clonotypic peptides. CH, constant part of heavy chain; CL, constant part of light chain; SS, disulfide bond; VH, variable part of heavy chain; VL, variable part of light chain.
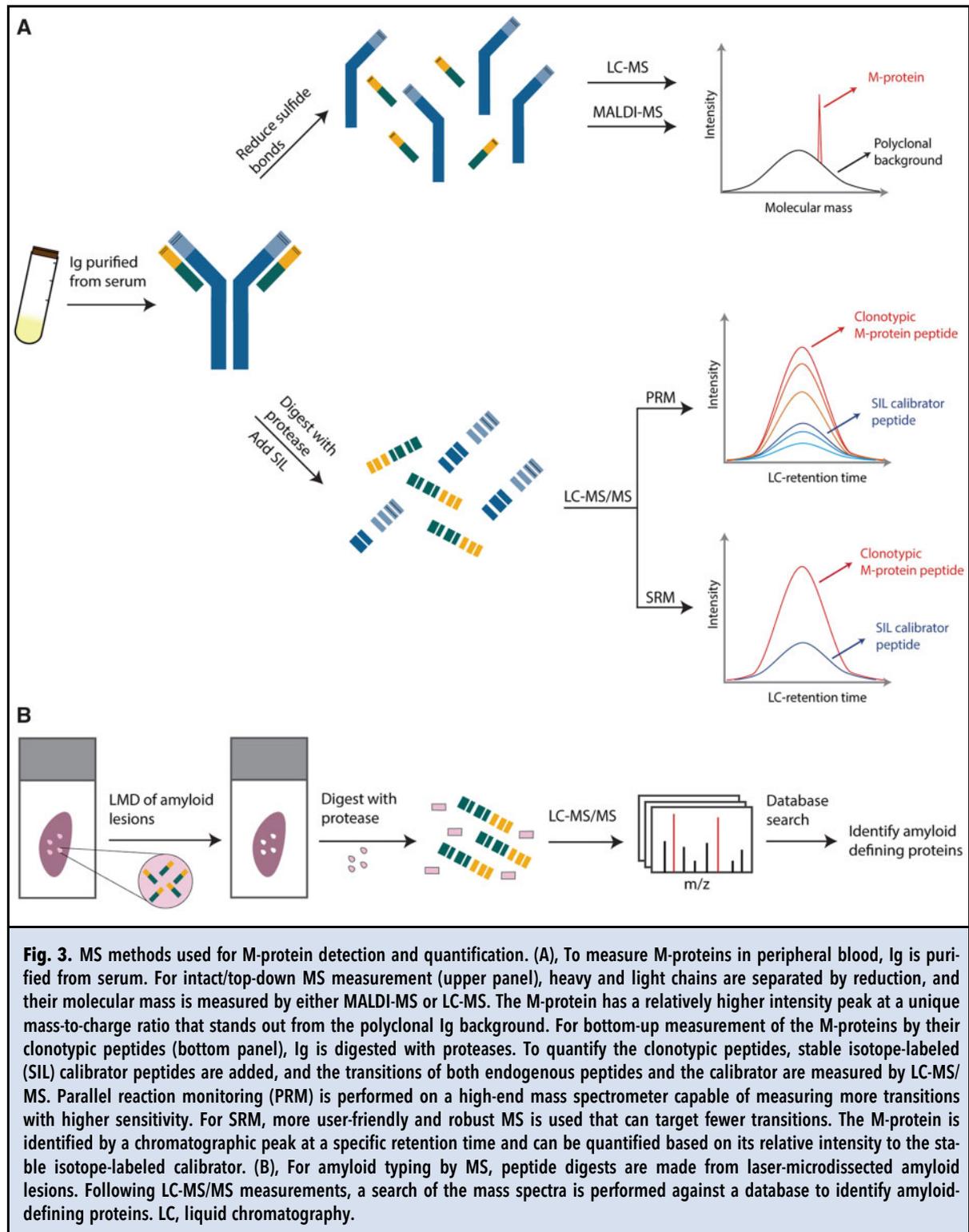
The chosen MS method and preanalytical process are dictated by the underlying clinical or research question. In the following paragraphs, we provide examples of how top-down MS can be used for high-throughput detection of an M-protein based on its unique mass; bottom-up MS can be used for highly sensitive quantification of an M-protein based on unique clonotypic peptides in the variable Ig region; and proteomics approaches can be used on tissue biopsy to detect monoclonal Ig deposits, for example, to diagnose AL amyloidosis.

M-Protein Quantification Using Intact Mass-Specific Methods (Top-down MS)

Intact protein analysis by MS (top-down MS) is based on monitoring the molecular mass of intact proteins or protein fragments (Fig. 3, A, upper panel). The application of intact protein analysis to M-protein diagnostics

is based on the unique molecular mass and high abundance of the monoclonal Ig distinguishable from the otherwise polyclonal background. This unique mass can be determined by MS and serves as a personalized biomarker to monitor patients. Sample processing is simpler for intact analysis compared with peptide-based methods because protein digestion is not necessary. The only required sample processing step is Ig isolation and further cleavage into Ig fragments. Ig isolation increases the analytical sensitivity due to decreased interference from other highly abundant proteins such as albumin (37).

The first evidence of feasibility of intact analysis to monitor M-proteins was described in 2014 by Barnidge et al. (38). In their proof-of-principle study, they isolated the M-protein from sera from a single patient with MM and dissociated the Ig into light and heavy chains (middle-down MS), which were then subjected to liquid

Fig. 3. MS methods used for M-protein detection and quantification. (A), To measure M-proteins in peripheral blood, Ig is puri-fied from serum. For intact/top-down MS measurement (upper panel), heavy and light chains are separated by reduction, and their molecular mass is measured by either MALDI-MS or LC-MS. The M-protein has a relatively higher intensity peak at a unique mass-to-charge ratio that stands out from the polyclonal Ig background. For bottom-up measurement of the M-proteins by their clonotypic peptides (bottom panel), Ig is digested with proteases. To quantify the clonotypic peptides, stable isotope-labeled (SIL) calibrator peptides are added, and the transitions of both endogenous peptides and the calibrator are measured by LC-MS/MS. Parallel reaction monitoring (PRM) is performed on a high-end mass spectrometer capable of measuring more transitions with higher sensitivity. For SRM, more user-friendly and robust MS is used that can target fewer transitions. The M-protein is identified by a chromatographic peak at a specific retention time and can be quantified based on its relative intensity to the sta-ble isotope-labeled calibrator. (B), For amyloid typing by MS, peptide digests are made from laser-microdissected amyloid lesions. Following LC-MS/MS measurements, a search of the mass spectra is performed against a database to identify amyloid-defining proteins. LC, liquid chromatography.

chromatography–quadrupole TOF analysis. Sequential monitoring of the unique mass of the M-protein light chain demonstrated improved analytical sensitivity compared with both gel-based techniques and FLC analysis. Furthermore, M-protein isotyping was possible because of the unique isotype-specific fragmentation pattern of the light chain. These proof-of-principle results led to further validation and development of the technique coined *miRAMM* (monoclonal Ig rapid accurate mass measurement) *(39)*. Subsequent studies revealed miRAMM to be a powerful tool in M-protein diagnostics and monitoring in matrices other than serum. A comparative study of light chain monitoring in urine by miRAMM and by IFE revealed equal analytical sensitivity of M-protein detection using miRAMM on neat urine and IFE on 200-fold concentrated urine (97.4% concordance) *(40)*. Further optimized versions of miRAMM demonstrated that this method can serve as a potential clinical assay that extends its analytical sensitivity for M-protein monitoring beyond that of conventional electrophoretic methods and the FLC assay. The superior analytical sensitivity of miRAMM was underscored in a study with 30 patients with MM reaching sCR after autologous stem cell transplantation *(41)*. At Day 100 after autologous stem cell transplantation, sCR samples had miRAMM identifiable M-proteins in 81% of patients, indicating increased analytical sensitivity compared with gel-based techniques. In this small data set, single time points of miRAMM M-protein status did not predict better progression-free survival. However, those patients whose miRAMM intensities decreased in 2 serial measurements had significantly longer progression-free survival compared with patients who did not experience decreased miRAMM intensities. The clinical relevance of miRAMM should be studied in a larger independent cohort with more frequent follow-up *(41)*. By analyzing nonreduced samples, miRAMM can also be applied for detection and quantification of monoclonal FLC, for example, for monitoring patients with light chain MM or AL amyloidosis *(42)*.

One drawback of miRAMM's limited use in high-volume routine diagnostics is the use of chromatography systems. Therefore, a chromatography-free system was developed by replacing micro Liquid Chromatography-Electrospray Ionization with MALDI *(43)*. This system allows higher sample throughput with measurement times of approximately 1 minute per patient sample. Moreover, the technique is simple in execution and has potential for automation. The combination of light chain immune enrichment and MALDI-TOF was termed *MASS-SCREEN* and provided a method to qualitatively screen for M-proteins in serum and urine. The clinical application of MALDI-TOF for M-protein monitoring in patient sera was demonstrated by Kohlhagen et al. *(44)*. In a comparative study performed

in >500 patients, the authors demonstrated that MASS-SCREEN could be a cost-competitive screening method to detect M-proteins with a detection capability comparable to that of IFE. Because FLC ratios were found to be abnormal in 28% of MASS-SCREEN–negative samples, the method cannot replace FLC immunoassays. MALDI-TOF with minimal preanalysis was recently also applied for rapid screening of monoclonal FLC in urine *(45)*.

A drawback of MASS-SCREEN is the inability to distinguish Ig isotypes because the Ig isolation is performed with κ- and λ-directed nanobodies. To also account for Ig isotypes while retaining the benefits of MALDI-TOF analysis, MASS-FIX was introduced *(46)*. In the MASS-FIX workflow, 5 Ig isolations are performed with nanobodies directed against the constant domains of the heavy chains (IgG, IgA, and IgM) as and the light chains (κ and λ). This isolation strategy and subsequent MALDI-TOF analysis of the different fractions enables combined identification, isotyping, and quantification of M-proteins. In a comparative study between MASS-FIX and routine M-protein diagnostics, it was shown that M-proteins in 98% of sera and 95% of urine samples were similarly isotyped by IFE and MASS-FIX. In this study, the capability for detecting a serum M-protein by MASS-FIX was at least equal to that of IFE. MASS-FIX quantification, with interassay CVs of <20% in most samples, provided equivalent quantitative information to SPE *(46)*. A separate study that included clinical samples across the entire spectrum of plasma cell disorders confirmed that MASS-FIX had a comparable capacity to detect an M-protein compared with IFE *(47)*. Increased detection of abnormal FLC ratios was accomplished by performing an additional immunoenrichment using Sepharose-coupled antibodies against FLC followed by MALDI-TOF MS *(48)*. This approach allows direct FLC detection and provides added confidence for diagnosing MG based on monoclonal FLC.

Atypical mass spectra observed using MASS-FIX may provide additional information on posttranslational M-protein modifications *(47)*. An interesting observation was that a relatively large proportion (16%) of patients with AL amyloidosis had atypical spectra caused by glycosylated clonal light chains. It was further shown that these glycosylated light chains were present years before the diagnosis of AL amyloidosis *(49)*. This could be an interesting feature to screen for the risk of asymptomatic patients with MG to progress into AL amyloidosis. The pathologic and clinical impacts of these modifications warrant further research. Modified top- and middle-down MS can provide broad sequence coverage, which enables extensive mapping and glycoprofiling of M-proteins *(50)*.

Taken together, MASS-FIX is potentially a powerful alternative to gel-based techniques in M-protein

diagnostics, with competitive semiautomated sample throughput and some clear analytical advantages. In 2018, MASS-FIX replaced IFE in routine clinical practice at the Mayo Clinic.

M-Protein Quantification Using Peptide-Specific Methods (Bottom-up MS)

Bottom-up MS using targeted proteomics methods have been developed for ultrasensitive M-protein monitoring in peripheral blood that can potentially compete with MRD testing in bone marrow aspirates. The clonotypic (also called proteotypic) approach to measuring M-protein is based on peptide-targeted MS performed on serum digests from MM patients (Fig. 3, A, lower panel). Peptides unique for patient M-protein are selected and targeted with selected reaction monitoring (SRM) or parallel reaction monitoring (28, 51, 52). Quantification of M-protein is possible by adding stable isotope-labeled peptides to serum or serum digest (53). Stable isotope-labeled peptides are selected from the clonotypic candidates after assessing their performance for sensitivity and selectivity.

Clonotypic peptide candidates may be deduced from patient DNA or RNA sequencing information of the clonal plasma cells in the bone marrow. The Ig sequences of the clonal plasma cells are aligned to Ig germline sequences, and peptides with mutations relative to the germline sequence are selected. Because of the V(D)J clonal rearrangements and somatic hypermutations in the Ig complementarity-determining regions, these sites are considered to be of most interest for clonotypic peptide selection. There are 3 complementarity-determining regions on both heavy and light chains in the Ig antigen-binding part. For sequencing, 1 bone marrow aspirate taken during active disease is necessary. Efforts to develop methodology that no longer requires bone marrow are ongoing (54). De novo sequencing on proteomics data may be feasible (55). Computational de novo sequencing, in which a full amino acid M-protein sequence would be constructed from experimental, high-resolution, MS data, could eliminate the need for genome information and bone marrow sampling if adequate reliability can be achieved (54, 55).

The detection capability of clonotypic targeted M-protein diagnostics is further improved by Ig purification during preanalysis to reduce the complexity of the patient serum. Digestion of the isolated Ig, including the M-protein, is most commonly performed with trypsin, and digested serum samples are measured on the mass spectrometer utilizing SRM (also called *multiple reaction monitoring*) (28, 51) and parallel reaction monitoring (52) technologies. SRM is usually performed with triple-quadrupole mass spectrometers to monitor targeted peptides and their selected fragments. Peptide

and fragment ion pairs are called *transitions*, and in SRM, the transitions with the highest signal intensity have to be selected for every targeted peptide (53). Conversely, parallel reaction monitoring is performed on high-resolution and high-accuracy mass spectrometers, and all fragments of targeted peptide can be detected in parallel, thus requiring less assay development than SRM (Fig. 3, A) (56). Although clonotypic peptides have the potential to offer superior sensitivity, the process is more laborious and time consuming compared with MS methods performed on reduced Ig fragments such as miRAMM. It is important to note that effectiveness of the clonotypic MS assay can vary in individual patients because the number of suitable clonotypic peptides and their performance is patient-specific.

Murray et al. (54) showed that clonotypic targeting is >1.000 times more sensitive than SPE quantification and has the potential to be more sensitive than MRD analysis performed on bone marrow aspirates. MRD analysis in bone marrow and MRD analysis on M-protein in serum both have potential weaknesses. For bone marrow–based methods, as mentioned earlier, a significant portion of patients with MM present with focused lesions. Such solitary lesions, or extramedullary disease, would go unnoticed in a bone marrow aspirate unless performed at the exact site of the lesion. Nonrepresentative sampling can strongly bias MRD quantification in bone marrow aspirates. In contrast, disease activity would go unnoticed in serum-based assays when performed in the rare event of patients in whom the MM clone does not secrete an M-protein (57). Furthermore, the M-protein is a surrogate marker of a cellular disease state. A confounding factor is the half-life of M-proteins in the blood: on average, 21 days for IgG and 10 days for IgA. This causes a delay between lysis of clonal plasma cells and the decrease in M-protein. It is challenging to compare the various MRD methods in terms of analytical performance because MFC measures myeloma cells, ASO-qPCR and NGS MRD techniques measure clonal DNA, and MS-based methods measure the M-protein. A good comparison between these methods for applicability, performance, and prognostic value is currently lacking.

MS Specifically Measures M-Protein without Interference from t-mAbs

The therapeutic landscape of MM has strongly evolved in the past decade. The first t-mAbs was approved for MM treatment, and a large list of biologics are being evaluated in clinical trials (58). Such t-mAbs are all human(ized) mAbs that can appear on electrophoretic scans as small monoclonal bands (59–61). In routine diagnostics, it may be challenging to differentiate the human(ized) t-mAbs from the endogenous M-protein.

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Consequently, the IMWG response criteria have been modified to account for the presence of t-mAb interference (62). However, comigration of t-mAbs and the endogenous M-protein can result in the inability to accurately assess therapeutic responses (61, 63). Electrophoretic interference of t-mAbs can be overcome using a biologic-specific antibody that binds the t-mAbs and shifts SPE migration. For daratumumab, a so-called shift assay has been realized (59). However, electrophoretic patterns will become increasingly difficult to interpret if multiple t-mAbs are combined for use in a single patient, and response assessment may not be possible.

MS methods can accurately quantify the M-protein without interference from multiple t-mAbs. Top-down MS makes use of the unique high-resolution mass of the t-mAbs (37, 64, 65). In fact, initial proof-of-concept work for miRAMM technology was performed by serial dilutions of adalimumab in normal human serum (38). In a recent study, miRAMM was able to correctly identify t-mAbs (daratumumab, elotuzumab, or isatuximab) and M-protein in 100% of the 192 samples tested (66). The chance for an M-protein to have a mass so close to the mass of the t-mAbs to cause interference in top-down MS is estimated to be small. The problem of t-mAb interference is also solved in the targeted MS workflow by merely adding unique t-mAb peptides to the assay for targeting (67). Our group has shown that M-protein can be detected in the presence of 3 additional t-mAbs without any cross-reactivity (52). By adding reference stable isotope-labeled peptides for the t-mAbs and for the M-protein, all can be quantified in a single assay to allow additional therapeutic drug monitoring.

MS of Affected Tissue for Detection of AL Amyloidosis

Amyloidosis is a life-threatening disease caused by extracellular deposition of insoluble fibrils that can affect a wide variety of organs. Many different proteins have been identified that form these pathogenic fibrils. Misfolded monoclonal light chains are the most common and cause AL amyloidosis (68). Accurate typing of amyloid is crucial for optimal treatment (68).

Traditionally, histologic diagnosis of AL amyloidosis is based on Congo red staining to confirm the presence of amyloidosis. Congo red binds to amyloid fibrils, and green birefringence is seen under polarized light. Immunohistochemistry of tissue biopsies, staining the various potential amyloid proteins, is performed for further amyloid typing (69). Immunohistochemistry using anti–light chain antisera can support confirmation of AL amyloidosis. However, immunohistochemistry interpretation is challenging because of the limited availability of type-specific antibodies, signal interference caused by tissue contamination from serum proteins, and false-negative results caused by the loss of epitopes in fixed tissue sections (69). Moreover, the size of the plasma cell clone in patients with AL amyloidosis is often modest, which may further complicate and delay diagnosis (70).

Proteomic typing of amyloid deposits using MS of affected tissue has not only improved amyloid diagnostics in terms of sensitivity and specificity but also has identified novel proteins as possible causes of amyloidosis (70). Proteomic amyloid typing is performed on samples collected by laser microdissection; that way, affected lesions enriched for amyloid deposits can be analyzed using MS (Fig. 3, B). A complete proteome amyloid signature for diagnosis and typing of amyloidosis can be made by MS-based proteomics on subcutaneous fat aspirates, which could replace Congo red staining for confirmation of amyloidosis (71, 72). The premise is that the protein causing amyloidosis represents a dominant protein in the deposits. Vrana et al. (73) have identified peptides from the constant regions of Ig in all cases of AL amyloidosis tested. Peptides from the variable regions can also be detected from patient MS data using an augmented database with light chain variable region sequence templates (74).

MS-based proteomics have to be performed in specialized centers because the experimental methods and data processing require experienced personnel. New efforts are aimed at amyloid typing by targeted MS utilizing SRM technologies on more clinical laboratory–friendly, lower resolution mass spectrometers, without laser microdissection (75). These methods target reference peptides for light chains to detect AL amyloidosis and heavy chains to control for serum contamination. The assays are multiplexed with other targets such as amyloid A and transthyretin amyloidosis to type amyloidosis with high sensitivity and specificity.

Conclusions

M-protein diagnostics can be challenging in individual patients because of patient-specific unique features. In addition, more sensitive assays are needed because improved treatment of patients with MG has resulted in deeper responses, with an increased number of patients who obtain sCR in which no disease activity is observed with routine M-protein diagnostics. Intact protein MS methods and clonotypic peptide MS methods have been developed that show promise for high-throughput M-protein detection and MRD measurements. These MS-based methods to measure M-proteins are applied on peripheral blood, which makes serial sampling possible to guide optimal personalized treatment. Consequently, they form an attractive alternative to the bone marrow–based methods currently applied for MRD detection (Table 1).

Although MS-based methods to measure M-proteins seem promising, several aspects have not been fully

addressed. First, current MS studies are based on relatively small sample sizes. Their feasibility and applicability in large cohorts have not yet been shown. Second, most studies have focused on MM and AL amyloidosis, with relatively little information on the applicability of MS-based methods in other MG, such as Waldenström's macroglobulinemia and plasmacytoma. Third, thresholds for sCR and MRD in peripheral blood using MS methods need to be defined. Finally, a direct comparison in which applicability, performance, prognostic value, and operational aspects such as cost and turnaround-time of MRD measured in bone marrow versus MS in peripheral blood has not yet been performed. MRD status obtained in bone marrow provides information that cannot be achieved by MS, such as clone evolution and bone marrow reconstitution. As such, we anticipate that, in the future, MS will not replace existing MRD tests in bone marrow but will have clinical value as a companion method, especially for monitoring of MRD in blood. This approach is in line with the recent IMWG recommendation (20) that the development of blood-based MRD monitoring should be the ultimate goal, as it would allow for serial sampling without the trauma of repeated bone marrow aspirations and ensures assessment of extramedullary disease, which is not evaluated by bone marrow biopsy.

Nonstandard abbreviations: MG, monoclonal gammopathy; Ig, immunoglobulin; M-protein, monoclonal immunoglobulin; MM, multiple myeloma; AL, amyloid light chain; FLC, free light chain; sCR, stringent complete response; MRD, minimal residual disease; t-mAb, therapeutic monoclonal antibody; MS, mass spectrometry; SPE, serum protein electrophoresis; IFE, immunofixation electrophoresis; IMWG, International Myeloma Working Group; MFC, multicolor flow cytometry; ASO-qPCR, allele-specific oligonucleotide–quantitative PCR; NGS, next-generation sequencing; miRAMM, monoclonal immunoglobulin rapid accurate mass measurement; SRM, selected reaction monitoring.

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