MULTIPLE MYELOMA

TOWARDS CLINICAL MONITORING OF MINIMAL RESIDUAL DISEASE USING MASS SPECTROMETRY

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Towards clinical monitoring of minimal residual disease using mass spectrometry

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Op weg naar het klinisch monitoren van minimale restziekte met massaspectrometrie

Thesis

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Contents

Chapter 1

Introduction

This chapter is adapted from Zajec M, Langerhorst P, VanDuijn MM, Gloerich J, Russcher H, van Gool AJ, Luider TM, Joosten I, de Rijke YB, Jacobs JFM. Mass Spectrometry for Identification, Monitoring, and Minimal Residual Disease Detection of M-Proteins. Clin Chem. 2020;66(3):421-33.

Monoclonal gammopathies

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

M-protein detection and quantification are integral parts of diagnosis and monitoring of MG.(2) M-protein may consist of intact monoclonal immunoglobulins (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 non-secretory 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 serum 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. Additionally, the introduction of therapeutic monoclonal antibodies (t-mAb) can directly hamper traditional M-protein diagnostics, since it may be challenging to distinguish the human(ized) t-mAb 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. As a result, M-protein has both 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 M-protein features, beyond the general region of electrophoretic migration. Mass spectrometry (MS) is ideally suited for accurate mass measurements or targeted measurement of unique M-protein peptides. It is therefore not surprising that new MS-based methods for the detection and sensitive quantification of M-proteins appeared in the literature beginning in 2014. Some of these novel methods have already been implemented in routine diagnostics. In the near future MS will play an increasingly important role in the field of M-protein diagnostics.

Routine M-protein diagnostics

M-protein is a serum biomarker that directly relates 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, as well as a quantitative biomarker for disease prognostication, to follow the course of disease, and to monitor response to therapy. M-protein diagnostics is performed using high resolution and semi-automated 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 (CE). 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-CE. Turbidimetric and nephelometric analyses are performed to quantify total IgG, IgA, IgM, FLC, and heavy-light chain pairs.(2, 3) Katzmann et al. 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.(6) 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 co-migrate with other abundant serum proteins, for example in the beta 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 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.(14)

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

Minimal residual disease 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 that achieve sCR, MRD assessment by multi-color flow cytometry (MFC), allele-specific oligonucleotide (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 sensitivity of 1 in $\geq 10^5$ nucleated cells.

Cellular (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. Characteristics of an ideal MRD assay are high sensitivity/specificity/reproducibility, feasibility for all MM patients, standardized among institutes, small sample volume, easily applicable, rapid turnaround time, and cost-effectiveness. None of the currently described methods to assess MRD meet all 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, since MRD kinetics over the disease course provides more robust evaluation of disease control in patients with MM after achieving sCR.(20)

The strongest limitation of the methods described above is that disease monitoring must be performed on bone marrow aspirates, which introduces the risk of non-representative 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 standardization challenging.(22) Besides this, the need for repetitive bone marrow punctures for patient follow-up is a physical burden that reduces the quality of life of an individual patient.

MRD evaluation in peripheral blood would represent an attractive minimally invasive alternative to circumvent the above mentioned disadvantages of MRD assessment in bone marrow. Studies investigating the possibility of detecting MM disease activity in peripheral blood have emerged that employ 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 circulating DNA are present at much lower levels

in peripheral blood compared to the bone marrow. Hence, 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.

Immunoglobulin measurements using mass spectrometry

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

Protein measurement using MS has been 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 to characterize proteins. More user friendly and more robust, newer generation MS-instruments have begun to play a role in clinical diagnostics.(26)

Liquid chromatography (LC)-MS is an analytical chemistry technique that combines the physical separation capacity of LC with the mass analysis capacity of MS. This technique can be used to analyse 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, as well as 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)

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.(30-32)

Mass spectrometry as a novel method for M-protein measurement in peripheral blood

Based on existing literature on analysis of Ig and t-mAb(33), 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 pre-analytical Ig processing into top-down, middle-down, and bottom-up. The intact Ig is the starting analyte in top-down MS, 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 analysed intact (middle-down) or after further digestion into peptides (middle-up).(30, 34)

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/digest these into fragments. Ig isolation decreases interference from other abundant serum proteins such as albumin. 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.(35) 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 that are 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 MM patients.

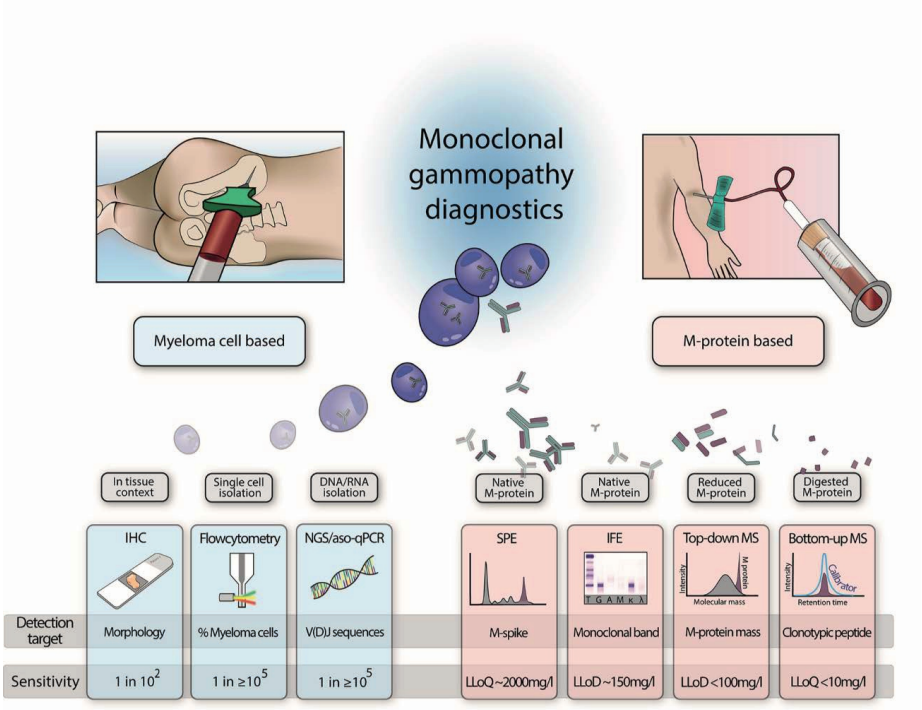


Figure 1. Graphical overview of individual features of a selection of techniques to monitor monoclonal gammopathy disease activity.

IFE, immunofixation electrophoresis; IHC, immunohistochemistry; LLoD, lower limit of detection; LLoQ, lower limit of quantification; MS, mass spectrometry; NGS, next generation sequencing; SPE, serum protein electrophoresis

M-protein quantification using peptide specific methods (bottom-up mass spectrometry)

Bottom-up MS using targeted proteomics has been developed for ultra-sensitive 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. Patient-specific M-protein peptides are selected and targeted in a selected reaction monitoring (SRM) or parallel reaction monitoring (PRM) fashion (Figure 2).(28, 36, 37) Quantification of M-protein is possible by adding stable isotope-labeled peptides to serum or serum digest.(38) Stable isotope-labeled peptides are selected from the clonotypic candidates after assessing their performance in sensitivity and selectivity.

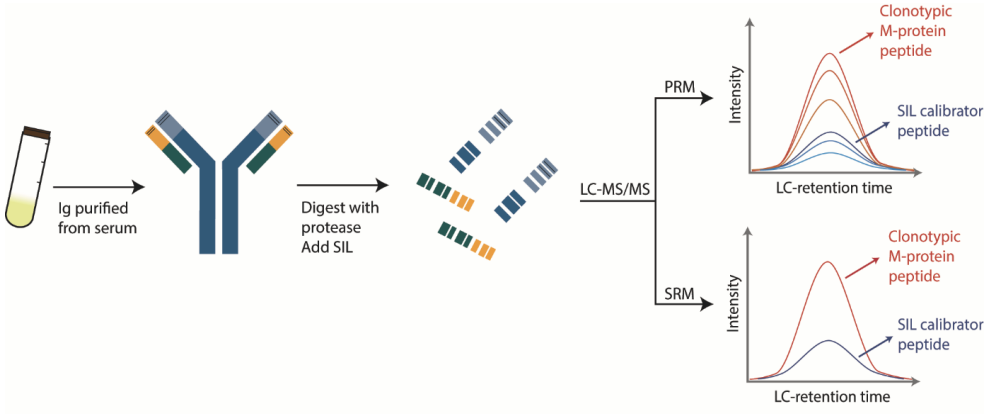


Figure 2. Bottom-up mass spectrometry for M-protein detection and quantification.

Ig, immunoglobulin; LC, liquid chromatography; MS, mass spectrometry; PRM, parallel reaction monitoring; SIL, stable isotope labelled; SRM, selected reaction monitoring

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 (CDRs), these sites are considered to be of most interest for clonotypic peptide selection. There are three CDRs on both heavy and light chain in the Ig antigen-binding part. For sequencing, one bone marrow aspirate taken during active disease is necessary. Efforts to develop methodology that no longer requires bone marrow are ongoing.(39) *De novo* sequencing on proteomics data may be feasible.(40) Computational *de novo* sequencing, in which 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.(39, 40)

The analytical sensitivity of clonotypic targeted M-protein diagnostics is further improved by Ig purification during pre-analysis 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, MRM) (28, 36) and PRM (37) 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.(38) Conversely, PRM is performed on high resolution and high accuracy mass spectrometers and all fragments of targeted peptide can be detected in parallel, therefore requiring less assay development than SRM.(41) It is important to note that effectiveness of the clonotypic MS-assay can vary in individual patients since the number of suitable clonotypic peptides and their performance is patient-specific.

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. Non-representative 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.(42) 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 which is 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 sensitivity since 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 on applicability, sensitivity and prognostic value is currently lacking.

Mass spectrometry measures M-protein without interference from therapeutic monoclonal antibodies

The therapeutic landscape of MM has strongly evolved in the past decade. The first t-mAb have been approved for MM treatment and a large list of biologics are being evaluated in clinical trials.(43) Such t-mAb are all human(ized) mAb that can appear on electrophoretic scans as small monoclonal bands.(44-46) In routine diagnostics it may be challenging to differentiate the human(ized) t-mAb from the endogenous M-protein. As a result, the IMWG response criteria have been modified to account for the presence of t-mAb interference.(47) However, co-migration of t-mAb and the endogenous M-protein can result in the inability to accurately assess therapeutic responses.(46, 48) Electrophoretic interference of t-mAb can be circumvented using a biologic-specific antibody that binds the t-mAb and shifts SPE migration. For daratumumab, a so-called shift-assay has been realized.(44) However, electrophoretic patterns will become increasingly difficult to interpret if multiple t-mAb 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-mAb. Top-down MS makes use of the unique high-resolution mass of the t-mAb.(49-51) Bottom-up, targeted, MS-workflow solves the problem of t-mAb interference by merely adding unique t-mAb peptides to the assay for targeting.(52) Our group has shown that M-protein can be detected in the presence of three additional t-mAb without any cross-reactivity.(37) By adding reference stable isotope-labeled peptides for the t-mAb as well as for the M-protein, all can be quantified in a single assay to allow additional therapeutic drug monitoring.

Aim and outline of this thesis

Aim of this thesis is to investigate possibilities for MM monitoring using state-of-the-art mass spectrometry technology. Electrophoretic methods for M-protein detection and characterization are routinely performed using serum samples, however, they lack in sensitivity. Bone marrow-based techniques, such as MFC, NGS and ASO-qPCR, are sensitive techniques for MRD detection, however uncomfortable for the patient and therefore not suited for repetitive patient monitoring. Using mass spectrometry the best of both worlds could be combined, that is highly sensitive MRD detection and minimally invasive serum-based sampling.

In **Chapter 2**, a targeted mass spectrometry assay is described for quantification of M-protein and three different t-mAbs. Combining the powers of proteomics and genomics a proof of concept was established for M-protein detection and quantification, in MM patient serum, using stable isotope-labeled peptides. We expanded on this proof of concept in **Chapter 3** and performed the assay on a

cohort of 23 MM patients. In **Chapter 4**, using the M-protein band from routine diagnostic electrophoretic gels as the starting material for mass spectrometry analysis was investigated.

The mass spectrometry assay requires DNA or RNA sequence for selection of patient-specific M-protein peptides for M-protein quantification, for that an initial bone marrow sample is necessary. To circumvent the need for bone marrow sampling, M-protein from 10 MM patients was monitored using *de novo* sequencing and mass spectrometry, making the assay completely serum-based (**Chapter 5**).

An interesting and rare case of a MM patient with leptomeningeal involvement is described in **Chapter 6**. This patient has developed leptomeningeal myeloma while on daratumumab maintenance therapy. Daratumumab was measured in cerebrospinal fluid and serum of this patient to explore the ability of daratumumab to cross the blood-brain barrier.

Finally, in **Chapter 7** the results of this thesis are discussed and summarized.

Chapter 2

Development of a targeted mass spectrometry serum assay to quantify M-protein in the presence of therapeutic monoclonal antibodies

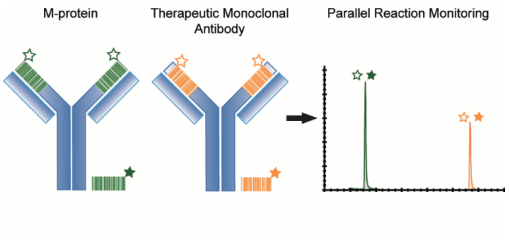
Abstract

M-protein diagnostics can be compromised for patients receiving therapeutic monoclonal antibodies as treatment in multiple myeloma. Conventional techniques are often not able to distinguish between M-proteins and therapeutic monoclonal antibodies administered to the patient. This may prevent correct response assessment and can lead to overtreatment.

We have developed a serum-based targeted mass spectrometry assay to detect M-proteins, even in the presence of three therapeutic monoclonal antibodies (daratumumab, ipilimumab and nivolumab). This assay can target proteotypic M-protein peptides as well as unique peptides derived from therapeutic monoclonal antibodies.

We address the sensitivity in M-protein diagnostics and show that our mass spectrometry assay is more than two orders of magnitude more sensitive than conventional M-protein diagnostics. The use of stable isotope labelled peptides allows absolute quantification of the M-protein and increases the potential of assay standardization across multiple laboratories.

Finally, we discuss the position of mass spectrometry assays in monitoring minimal residual disease in multiple myeloma, which is currently dominated by molecular techniques based on plasma cell assessment that requires invasive bone marrow aspirations or biopsies.



Introduction

Plasma cells are differentiated B cells that secrete antibodies. In multiple myeloma (MM) a monoclonal population of plasma cells starts to proliferate in an uncontrolled way. These clonal plasma cells produce a monoclonal immunoglobulin called M-protein.(53) Each M-protein is obtained by gene rearrangement, somatic hypermutation and class switching processes. Therefore, the M-protein is unique to the patient and can be used as a marker for personalized cancer diagnostics and monitoring. In this technical note we present a mass spectrometry assay that allows absolute quantification of multiple monoclonal immunoglobulins in serum samples of MM patients.

Therapeutic monoclonal antibodies (mAbs) are promising agents for treatment of MM. They have shown to improve depth and duration of response in MM patients.(54-56) However, their use in patients introduces additional monoclonal antibody in the blood. The International Myeloma Working Group (IMWG) has defined criteria for response to treatment in MM.(7) These include changes in M-protein levels using serum protein electrophoresis (SPE) and immunofixation electrophoresis (IFE). Therapeutic mAb are detected with SPE/IFE and may be misinterpreted as an M-protein.(44, 45) The IMWG criteria to achieve complete response require, amongst others, no detectable M-protein by SPE/IFE. In this respect, therapeutic mAb interference may have clinical impact on the response assessment and may result in underestimated response rates of MM patients treated with these therapeutics.(44, 45)

Daratumumab specific IFE Reflex Assay (DIRA) was developed to circumvent interference of daratumumab, a therapeutic mAb used in multiple myeloma treatment.(44) DIRA uses daratumumab specific antibody that binds daratumumab and shifts migration in electrophoresis making it feasible to distinguish between endogenous M-protein and therapeutic mAb. DIRA is useful only for patients receiving daratumumab. If multiple therapeutic mAbs are administered to a patient, SPE/IFE patterns become increasingly difficult to interpret and response assessment may not be possible. The clinical study of nivolumab combined with daratumumab with or without lenalidomide-dexamethasone in relapsed and refractory MM is an example in which combinations of therapeutic mAbs are administered to MM patients and M-protein diagnostics will be challenging in this study.(57) It has been emphasized by van de Donk et al. that, as more therapeutic mAbs and their combinations get approval, it will be crucial to distinguish M-protein and therapeutic mAbs.(45)

Improved treatment strategies for MM lead to a larger number of patients obtaining complete remission in which residual tumor load can no longer be detected.(58, 59) Unfortunately, after this temporary improvement most of patients eventually relapse as a small number of cancer cells remains when the patient is in complete remission, this is called minimal residual disease (MRD).(60) Serum electrophoresis is not sensitive enough to detect MRD in MM patients, a more sensitive next step is

plasma cell assessment in the bone marrow.(61) Sensitive assays to detect MRD, e.g. flow cytometry, allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) and next generation sequencing (NGS) of the immunoglobulin gene rearrangements, are all performed on invasively sampled bone marrow aspirates/biopsies.(62) Efforts to monitor M-protein in serum led Mayo Clinic scientists to develop several different proteomic based approaches.(36, 39, 63) Mills et al. have detected the M-protein by determining the unique mass of the monoclonal immunoglobulin light chain and monitored patients in remission.(63, 64) Another approach was shown by Barnidge et al.; they have used peptides from the variable region of the immunoglobulin heavy chain to show the possibility to detect M-protein in serum of a MM patient.(36)

We have developed a targeted mass spectrometry (MS) assay to detect M-protein in serum of a MM patient in the presence of therapeutic mAbs. Similar to Barnidge et al. we explore the use of targeting proteotypic peptides from rearranged variable regions of the M-protein. We add stable isotope labelled (SIL) peptides, as internal standards for absolute quantification, to increase reliability and improve sensitivity of the assay. We also add proteotypic peptides derived from the therapeutic mAbs to the assay. Utilizing high resolution and mass accuracy of the Orbitrap Fusion Lumos, in one MS run M-protein and therapeutic mAbs can be detected and quantified. In addition, increased sensitivity of the MS assay compared to conventional M-protein diagnostics allows monitoring of deep remissions.

Experimental Section

Biological material

Serum of a MM patient, from which a bone marrow sample and heavy chain Sanger sequencing data is available, was chosen to determine the sensitivity of the assay. Specificity of the assay was tested on 23 sera including three sera from amyloid light chain (AL) amyloidosis patients, 13 MM patients and seven sera from healthy individuals. All serum samples and clinical data from AL amyloidosis patients, MM patients and healthy individuals were coded and anonymized as specified in the Dutch code of conduct for biomedical research.

Determination of immunoglobulin heavy chain (IGH) variable, diversity and joining (V(D)J) nucleotide sequence

To identify the clonal IGH-V(D)J rearrangement, clonality assays were performed on the DNA extracted from bone marrow through the amplification of IGH-VJ (FR1) and the IGH-leader using the multiplex BIOMED-2 PCR protocol.(65) PCR products were monitored through fluorescent gene scan analysis on an ABI 3730 platform (Thermo Fisher Scientific/Life Technologies, Foster City, CA). Sanger sequencing of the clonal IGH-VDJ rearrangement was performed, using at least two independently generated PCR products by the consensus JH primer and subsequently with the respective IGH-V

primer. Sequencing was performed on an ABI 3730 platform (Thermo Fisher Scientific/Life Technologies). The IGH-V(D)J nucleotide sequences were aligned using the IMGT-IgBLAST database.(66, 67) Homology to the nearest germline IGHV gene was calculated starting at the first nucleotide 3' from the FR1 forward primer to the last codon (CDR3-IMGT codon 105/106/107, depending on exonuclease trimming) of the IGHV gene.(68) Care was taken that length of the sequenced PCR fragment corresponded to the length evaluated by gene scanning.

Therapeutic monoclonal antibodies

Aliquots of daratumumab (Janssen Biotech, Leiden, The Netherlands), ipilimumab (Brystol-Myers Squibb, Utrecht, The Netherlands), and nivolumab (Brystol-Myers Squibb, Utrecht, The Netherlands) were taken from remainders of solutions for injections.

Proteotypic peptides

Primary sequences of M-protein and therapeutic mAbs were aligned to the most homologous germline variable region of the IMGT reference directory (http://www.imgt.org/). Tryptic peptides with mutations in the amino acid sequence compared to the germline reference were considered as proteotypic peptide candidates for M-protein and therapeutic mAbs and assessed for signal intensity in a shotgun proteomics experiment. Two proteotypic peptides were chosen for M-protein, one peptide for nivolumab, one for daratumumab, and one for ipilimumab (Table 1). SIL peptides (Pepscan B.V., Lelystad, The Netherlands) were used as internal standards for quantification of the M-protein and therapeutic mAbs. Peptide amount and purity were established by amino acid analysis, high-performance liquid chromatography (HPLC) and MS analysis by the manufacturer. Peptides were delivered as 5 µM solution in 5% acetonitrile/water.

Table 1. Proteotypic peptides used for quantification of M-protein and therapeutic monoclonal antibodies. Mutations relative to the most homologous germline sequence are shown in bold and underlined.

Protein	**M-protein peptide sequence**	**Germline peptide sequence**
Daratumumab	GLEWVSAISGSGG**G**TYYADSVK	GLEWVSAISGSGGSTYYADSVK
Ipilimumab	TGWLG**P**FDYWGQGTLVTVSSASTK	TGWLGYFDYWGQGTLVTVSSASTK
M-protein	GLEWVSYISS**G**GGSTYYADSVK	GLEWVSYISSSGGSTYYADSVK
M-protein	NSL**S**LQMN**N**LR	NSLYLQMNSLR
Nivolumab	ASG**I**TFS**NS**GMHWVR	ASGFTFSSYGMHWVR

The MMRF CoMMpass Study℠

Multiple Myeloma Research Foundation (https://www.themmrf.org/) provides access to data that result from the CoMMpass study on the NIH dbGAP platform (accession phs000748.v6.p4). The

database has a collection of RNA and DNA sequencing data from MM patients. We used this database to test if we can find M-protein proteotypic peptides for 20 randomly chosen MM patients with available RNA sequencing data. Raw data files are in the SRA data format in the database. Fastq-dump (SRA Toolkit, NIH) was used to convert SRA data into fastq format. As the sequence depth exceeds largely the requirements for our analysis, a random 1% subset of reads was extracted with famas (https://github.com/andreas-wilm/famas) and that subset was the input material for Targeted Assembly of Short Sequence Reads (TASR).(69) Additional input for TASR were sequence seeds which are sequences of constant domains of every type of immunoglobulin heavy and light chain. Seeds are used as a primer sequence on which the M-protein sequence contig is extended based on the copy number of the reads in the dataset. The nucleotide sequence of the M-protein that was a result from TASR was translated to an amino acid sequence and aligned to the closest germline variable region of the IMGT domain directory. M-protein was digested in silico (http://web.expasy.org/peptide_mass/) with trypsin, and we searched for peptides with mutations in the amino acid sequence and with length close to the optimal length for sensitive and selective detection (7-15 amino acids) by MS.

Sample preparation and immunoglobulin (Ig) purification

Equal volumes (2 µL) of MM patient serum and SIL peptide solutions of M-protein and therapeutic mAbs proteotypic peptides were mixed and diluted 250 times in 50 mM ammonium bicarbonate containing 20% acetonitrile. This material was used directly for digestion for proteomic analysis. In addition, Ig was purified from MM patient serum (10 µL) before adding SIL peptides. Melon Gel (Pierce, Rockford, IL) resin was used to purify Igs from serum according to the manufacturer's protocol (Melon purified Ig). Equal volumes (10 µL) of the Melon purified Ig and 10 times diluted SIL peptides were mixed and diluted 3 times in 20% acetonitrile.

Digestion of samples for proteomic analysis

To 30 µL of diluted serum or Melon purified Ig, equal volume of 0.2% Rapigest SF (Waters, Milford, MA) was added. In the next step, proteins were reduced and alkylated by adding dithiothreitol (DTT) to a final concentration of 10 mM - with a 30 minutes incubation period at 60°C and iodoacetamide (IAA) to the final concentration of 14 mM - with a 30 minutes incubation period at room temperature in dark, respectively. Then, pH was checked to be around 8 and subsequently 400 ng of gold-grade trypsin (Promega, Madison, WI) were added per sample for overnight incubation at 37°C. Digestion was terminated by adding trifluoroacetic acid (TFA) to a pH < 2 and incubating samples 30 minutes at 37°C. Samples were then centrifuged for 35 minutes at 20000 g at 4°C and the supernatant was used for preparing dilutions.

Dilution series preparation for assay sensitivity estimation

In order to mimic MRD, MM serum was diluted into a healthy control serum matrix containing therapeutic mAbs in concentration of 1 g/L for daratumumab and 0.5 g/L for ipilimumab and nivolumab. Control serum matrix with therapeutic mAbs was prepared and digested in the same way as MM serum and Melon purified Ig. Using control serum that did not contain M-protein, 10 dilutions with 5 fold incremental steps were prepared.

Liquid chromatography-mass spectrometry (LC-MS) measurements

LC was carried out on a nano-LC system (Ultimate 3000, Thermo Fisher Scientific, Munich, Germany). Samples were separated on a C18 column (C18 PepMap, 75 µm ID × 250 mm, 2 µm particle and 100 Å pore size; Thermo Fisher Scientific) and peptides were eluted with the following binary linear gradient of buffer A and B: 4%–36% solvent B in 28 minutes. Solvent A consists of 0.1% aqueous formic acid in water and solvent B consists of 80% acetonitrile and 0.08% aqueous formic acid.

Parallel reaction monitoring (PRM) measurements were performed on an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA). For electrospray ionization, nano ESI emitters (New Objective, Woburn, MA) were used and a spray voltage of 1.8 kV was applied. The instrument was operated in a targeted MS/MS using following parameters: quadrupole isolation width of 0.4 *m/z*, HCD fragmentation, MS/MS AGC target of 50,000 at a maximum injection time of 246 ms, and orbitrap resolution of 120,000. MS/MS spectra were collected in centroid mode. Parent masses, charge states, fragment masses and fragment types (b and y ions) are listed in Table S-2 in the Supporting Information. We used a scheduled method with a 144 second measurement window around the expected retention time.

Data processing

Signals were integrated using Skyline.(70) Concentration of each peptide was calculated from the peak area ratio between the endogenous and the SIL peptides, except where mentioned otherwise.

Conventional M-protein analysis

SPE and IFE were performed on a Hydrasys device (Sebia, Evry, France) using reagents from Sebia. Serum Free Light Chain analysis was performed using the Freelite FLC assay (The Binding Site, Birmingham, UK) on a BNII analyzer (Siemens, Marburg, Germany). SPE, IFE and FLC assay were all performed according to manufacturer's protocols. MM serum was diluted into control serum matrix, and 5 fold dilutions were prepared and analyzed.

Specificity

Melon purified Ig samples of 23 different control sera were collected, digested with trypsin and measured with the same PRM method as mentioned above. In order to show that peptides chosen for targeting are proteotypic for M-protein and therapeutic mAbs, specificity of peptides was assessed by determining whether a signal can be detected in the samples of control sera.

Sensitivity

Three independent runs of serum and Melon purified Ig dilution series were measured. Sensitivity was determined for each peptide separately. Limit of detection (LOD) was defined as ($3.3xSD_{control\ serum}$)/slope, and lower limit of quantification (LLoQ) was defined as 3xLOD, according to ICH guidelines (http://www.ich.org).

Results

Extracting proteotypic peptides from RNA sequencing data – the CoMMpass study

From the CoMMpass study we have randomly selected 20 different MM patients to show the feasibility of finding M-protein proteotypic tryptic peptides from RNA sequencing data from bone marrow samples. For 19 of the 20 patients we were able to find two or more predicted proteotypic tryptic peptides, for one patient we were able to find only one proteotypic tryptic peptide. Seven patients had light chain MM, and 13 patients had an M-protein consisting of both heavy and light chains. In Table S-1 in the Supporting Information, we show M-protein proteotypic tryptic peptides for a subset of five representative MM cases (two cases with light chain MM).

Specificity

For the M-protein proteotypic peptide GLEWVSYISSGGGSTYYADSVK no signals corresponding to peptide transitions were identified in the samples of control sera. For the other M-protein proteotypic peptide, NSLSLQMNNLR, we observed similar distribution of transitions in the control serum and in the MM serum. However, intensity of these transitions in control serum amounted to less than 0.1% of that in MM serum.

No signals corresponding to therapeutic mAbs proteotypic peptide transitions were identified in the samples of MM patient serum and control sera.

Sensitivity

To demonstrate the difference in sensitivity between conventional M-protein diagnostics and MS we have measured MM serum in various dilutions in control serum by SPE, IFE and FLC assay. Because of already mentioned interference of therapeutic mAbs with M-protein in conventional M-protein diagnostics, also illustrated in Figure S-1. in the Supporting Information, SPE and IFE were performed without adding therapeutic mAbs. M-protein concentration in the undiluted MM serum was determined to be 16.6 g/L by densitometry, and the M-protein was characterized by IFE as immunoglobulin G with a kappa light chain (IgG-κ). Figure 1 shows the SPE and IFE results of the first four M-protein dilutions. In the 125 times diluted serum (130 mg/L of M-protein) SPE/IFE can no longer detect the M-protein. Also, the FLC ratio normalizes in the 125 times diluted serum.

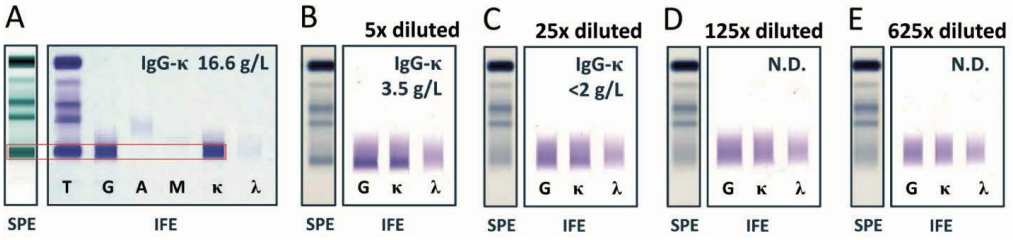


Figure 1. Serum protein electrophoresis (SPE) and immunofixation electrophoresis (IFE) scans of the undiluted multiple myeloma (MM) patient serum (A) and 4 dilutions in control serum (5, 25, 125, 625 times diluted MM serum correspond to B, C, D, E, respectively). M-protein concentration in the undiluted MM serum was determined to be 16.6 g/L by densitometry, and the M-protein was characterized by IFE as immunoglobulin G with a kappa light chain (IgG-κ). The concentration and class characterization are annotated on each gel. Red rectangle in the scan of the undiluted multiple myeloma serum illustrates immunoglobulin band, in the gamma region of the electrophoresis, that is indicative of the disease. The M-protein is no longer detectable with SPE and IFE in the 125 times diluted sample as there is no detectable band in the gamma region. N.D. = not detectable.

Dilution series with the M-protein in the presence of therapeutic mAbs was measured by MS and sensitivity was calculated for each M-protein proteotypic peptide separately. These two peptides show different sensitivity which is shown in Figure 2; LOD and LLoQ were calculated for both peptides (Table 2). We have also measured dilution series of therapeutic mAbs to estimate LOD and LLoQ for daratumumab, ipilimumab and nivolumab (Table 2). For both M-protein proteotypic peptides, detection and quantification in Melon purified Ig was more sensitive than in serum. LLoQ in serum was 6.2 and 13.7 mg/L, and LLoQ in Melon purified Ig was 0.7 and 0.8 mg/L for GLEWVSYISSGGGSTYYADSVK and NSLSLQMNNLR, respectively.

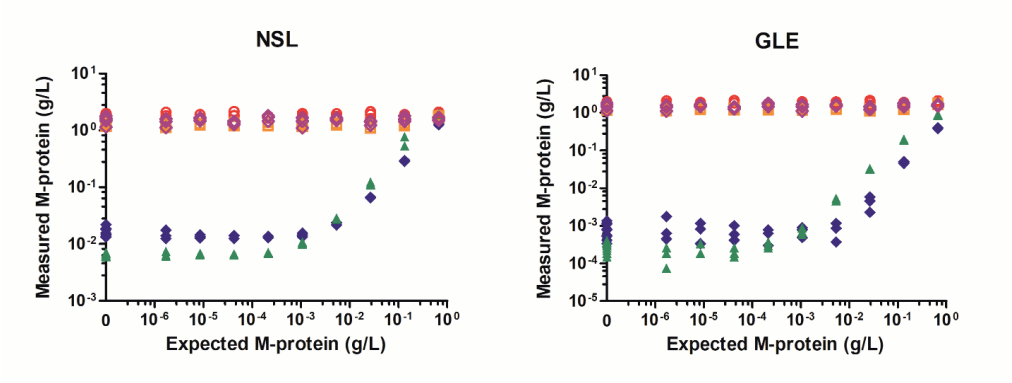


Figure 2. Dilution series (starting from 25 times diluted multiple myeloma serum and Melon purified Ig) for two M-protein proteotypic peptides, NSL – NSLSLQMNNLR, GLE – GLEWVSYISSGGGSTYYADSVK. Data points in blue diamonds describe dilution series of multiple myeloma serum in a control serum matrix, and green triangles describe Melon purified Ig dilution series. Data points in red circles, orange squares and purple diamonds represent spiked-in levels of daratumumab, ipilimumab and nivolumab, respectively. Control serum matrix data points, without M-protein, are plotted at 0.

Table 2. Limit of detection (LOD) and lower limit of quantification (LLoQ) values for M-protein and therapeutic monoclonal antibodies proteotypic peptides; quantified in serum using stable isotope labelled peptides.

Protein	Serum		Melon purified Ig	
	LOD (mg/L)	LLoQ (mg/L)	LOD (mg/L)	LLoQ (mg/L)
Daratumumab	0.3	0.9	0.3	0.9
Ipilimumab	0.2	0.7	0.1	0.4
M-protein (GLE*)	2.1	6.2	0.2	0.7
M-protein (NSL*)	4.5	13.7	0.3	0.8
Nivolumab	0.6	1.7	0.3	0.9

*GLE = GLEWVSYISSGGGSTYYADSVK, NSL = NSLSLQMNNLR

With MS the M-protein can still be detected in 15625 times diluted MM serum (1 mg/L of M-protein) in the presence of therapeutic mAbs. The developed MS assay can target M-protein and therapeutic mAbs and it is more than two orders of magnitude more sensitive than conventional M-protein analysis (Figure 3).

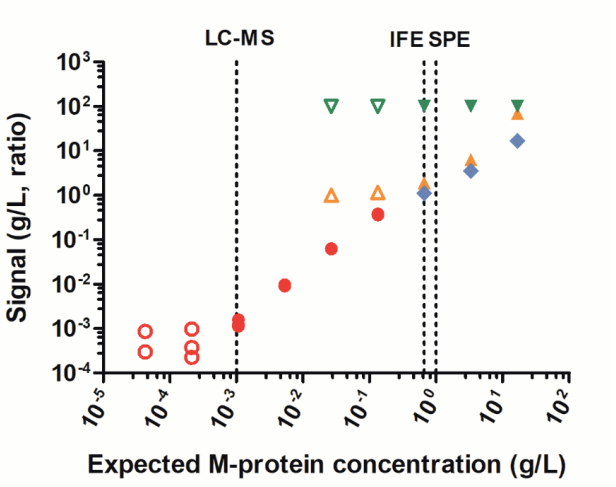


Figure 3. Comparison of the data obtained from M-protein dilution series measured by liquid chromatography-mass spectrometry (LC-MS), serum protein electrophoresis (SPE), immunofixation electrophoresis (IFE) and Freelite assay.
Dotted lines indicate M-protein detection limits for LC-MS, IFE and SPE, respectively. Full shapes show LC-MS/SPE/IFE/Freelite ratio signals indicative of disease, and empty shapes indicate negative signal (no M-protein detected). Red circles indicate LC-MS data points in triplicates from Melon purified Ig series for peptide GLEWVSYISSGGGSTYYADSVK, same as in Figure 2. Orange triangles indicate data points from Freelite assay. Blue diamonds indicate SPE data points and inverted green triangles indicate IFE. IFE results are not quantitative therefore they are included at numerical value 100.

We have also calculated LOD and LLoQ for peptide GLEWVSYISSGGGSTYYADSVK in Melon purified Ig dilution series without utilization of information from SIL peptides. In this case, calculations were based on the concentration of the M-protein known from the SPE measurement of the undiluted patient sample. From the peak area ratio for the endogenous peptide between this and the subsequent samples we could derive the concentration of M-protein in all dilutions. Results show slightly inferior sensitivity, compared to using SIL peptides, with LOD = 0.5 mg/L (was 0.2 mg/L with SIL peptide) and LLoQ = 1.5 mg/L (was 0.7 mg/L with SIL peptide). Another advantage of using SIL peptides can be seen in Figure 4. If we look at extracted fragment ion signals for peptide GLEWVSYISSGGGSTYYADSVK in 25 times diluted MM serum (700 mg/L of M-protein), which represents still relatively high concentration of M-protein that can even be detected by SPE, there is no doubt that the peak represents fragments for the endogenous peptide. However, in 625 times diluted serum (30 mg/L of M-protein, Figure 4A), if not using SIL peptides, a noisy peak with almost the same retention time can disturb the confidence in selecting the appropriate chromatographic peak.

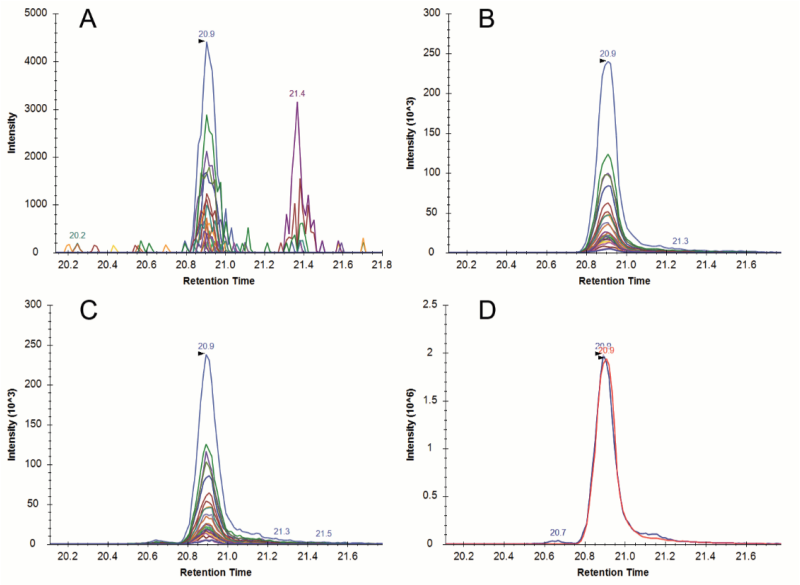


Figure 4. Extracted fragment ion signals for the endogenous peptide GLEWVSYISSGGGSTYYADSVK in 625 times diluted sample (A) and 25 times diluted sample (B); for the stable isotope labelled heavy form of the peptide in 25 times diluted sample (C); and integrated signals for the endogenous and heavy peptide in 25 times diluted sample (D) in red and blue, respectively. Retention time of the most intense transition (fragment ion data) are shown above the chromatographic peak.

Discussion

In this technical note we have shown feasibility to detect and quantify M-protein in serum of a MM patient in the presence of three different therapeutic mAbs. This MS assay circumvents the interference coming from administered monoclonal antibodies because it targets proteotypic peptides from the variable regions of immunoglobulins. Clinical studies suggest a possible benefit from the use of multiple therapeutic mAbs for treatment of MM.(57) If multiple therapeutic mAbs are administered to a patient, SPE and IFE patterns become difficult to interpret and response assessment may not be possible. Interference in conventional diagnostics can be circumvented using DIRA for daratumumab, and similar tests for other mAbs.(44, 45) However, even if the results of DIRA are negative, which means absence of M-protein, more sensitive tests (e.g. flow cytometry, ASO-PCR, NGS technologies) are used to measure MRD.(21, 71) Flow cytometry assays may also suffer from interference of mAb therapy where the mAb epitope overlaps with the markers used for staining in the assay, as highlighted for CD38 and daratumumab.(72, 73)

Ideally, proteotypic peptides for targeting in PRM are 7 – 15 amino acids in length. We have selected two M-protein proteotypic peptides. Peptide NSLSLQMNNLR has two mutations and 11 amino acids.

In theory it should be a better candidate, having more mutations and a shorter amino acid sequence. The background signal that we observed in control sera (less than 0.1% of the signal in MM serum) might be causing inferior sensitivity. It is not known whether the peptide is present in control sera in small amounts or whether it is another peptide that mimics the proteotypic peptide. Additionally, because of the presence of methionine, we have monitored the oxidized form of this peptide. Signals from the oxidized form of the peptide amounted to 1-2% compared to the non-oxidized form. Peptide GLEWVSYISSGGGSTYYADSVK has one mutation and 22 amino acids which is relatively long for PRM. For this peptide no interferences were seen in control sera and it was more sensitive than NSLSLQMNNLR for quantifying M-protein.

To show feasibility to select M-protein proteotypic peptides for additional patients we have used RNA sequencing data from 20 patients in The MMRF CoMMpass Study. Seven patients had a light chain only M-protein, which lowers the number of possible peptide candidates, as heavy chains are completely missing. We were able to find potential tryptic proteotypic peptides for all 20 patients which shows that finding proteotypic peptides for different MM patients is feasible.

Measurements of M-protein dilutions in a control serum show two orders of magnitude difference in sensitivity in favor of MS vs. SPE. Detection of the M-protein was more sensitive after immunoglobulins were purified from serum using Melon gel extraction. This probably relates to the reduction of the complexity of the serum matrix after immunoglobulin purification, which reduces ion suppression and enables the introduction of an increased amount of immunoglobulins into the mass spectrometer. As current M-protein diagnostics has limited sensitivity, patient follow-up would benefit from more sensitive assays that allow MRD assessment. New techniques to measure MRD all focus on assessment of clonal plasma cells present in the bone marrow.(62) On the other hand, MS results report the concentration of the M-protein in patient serum. It is difficult to compare such different metrics. For positioning MS in defining responses in MM, future work needs to investigate how these types of information potentially complement each other.

Bergen et al. have developed an MS assay to detect M-protein in patient serum and measured samples from MM patients that were negative by conventional flow cytometry.(39) From 10 patients that had no detectable disease by flow cytometry, all 10 had detectable disease by MS. They have shown that MS is sensitive enough to compete with conventional flow cytometry. Efforts to improve the sensitivity of conventional flow cytometry led scientists of the EuroFlow consortium to develop novel next generation flow (NGF) for highly sensitive MRD detection in MM.(73) They have improved sensitivity by optimizing sample preparation, antibody panels and software tools used for plasma cell gating; and they have shown that 25% of patients that were MRD negative by conventional flow cytometry, were MRD positive by NGF. However, they report discordance in assigning MRD

negative/positive status by NGF and NGS in 30% of cases. This indeed shows that one size does not fit all in MRD testing in MM and emphasis should be on understanding how results of different techniques for MRD testing relate to each other.

Standardization of targeted MS assays across multiple laboratories is possible through the use of SIL peptides as internal standards.(74) Using SIL peptides increases sensitivity and reliability, especially at lower M-protein concentrations. Sensitive M-protein quantification has to be reliable at the diagnosis, when the tumor load and the concentration of the M-protein are high, and also at MRD when the concentration of the M-protein is very low. We are confident that the correct peak is measured because endogenous peptides corresponding to the M-protein and SIL peptides elute at the same retention time. We have added the SIL peptides to the samples before digestion, as SIL peptides correct for experimental and instrumental variability, and for potential loss of the endogenous peptide caused by sample preparation or peptide adsorption on surfaces.(38)

Mills et al. suggested that the turnaround time to adapt the assay for clinical application would be a bottleneck in the proteotypic approach, as opposed to measuring the intact light chain.(64) However, quick and cheap SPE and IFE remain the gold standard in M-protein diagnostics. Therefore, we envision applying the MS assay to measure lower levels of M-protein, after treatment, when the advantages offered by SIL peptides, i.e. standardization and absolute quantification, overcome this turnaround time.

In the context of patient comfort, serum sampling is less invasive than bone marrow biopsy, therefore a serum-based assay is a desirable alternative for disease monitoring. Additionally, bone marrow based approaches introduce risk of sampling error coming from tumor heterogeneity. For the developed MS serum-based assay repeated bone marrow aspirations are not necessary during patient follow-up. DNA/RNA sequence is necessary for extracting proteotypic M-protein peptides which are key elements of the assay, therefore one bone marrow sample was used for sequencing. Potentially, M-protein sequence could be acquired from peripheral blood instead from the bone marrow, either from MM cells circulating in peripheral blood or by de novo sequencing based on serum immunoglobulin MS data.(39, 75-77)

Conclusion

The developed mass spectrometry assay can circumvent repeated bone marrow aspirations, enables simultaneous absolute quantification of M-protein and therapeutic monoclonal antibodies and is more than two orders of magnitude more sensitive than conventional M-protein diagnostics. This technical note shows the feasibility of the approach and has to be validated on longitudinally collected samples from a cohort of multiple myeloma patients.

Acknowledgements

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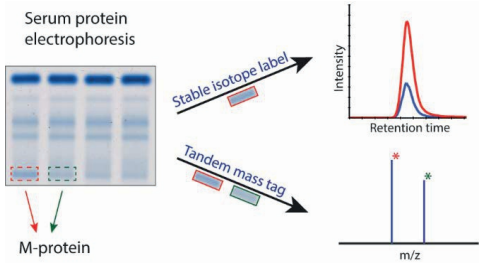
Chapter 4

Integrating serum protein electrophoresis with mass spectrometry, a new workflow for M-protein detection and quantification

Abstract

Serum protein electrophoresis (SPE) and immunofixation electrophoresis (IFE) are standard tools for multiple myeloma (MM) routine diagnostics. M-protein is a biomarker for MM that can be quantified with SPE and characterized with IFE. We have investigated combining SPE/IFE with targeted mass spectrometry (MS) to detect and quantify the M-protein. SPE-MS assay offers the possibility to detect M-protein with higher sensitivity than SPE/IFE, which could lead to better analysis of minimal residual disease in clinical laboratories. In addition, analysis of archived SPE gels could be used for retrospective MM studies. We have investigated two different approaches of measuring M-protein and therapeutic monoclonal antibodies (t-mAbs) from SPE/IFE gels. After extracting proteotypic peptides from the gel, they can be quantified using stable isotope labeled (SIL) peptides and measured by Orbitrap mass spectrometry. Alternatively, extracted peptides can be labeled with tandem mass tags (TMT). Both approaches are not hampered by the presence of t-mAbs. Using SIL peptides, limit of detection of the M-protein is approximately 100-fold better than with routine SPE/IFE. Using TMT labeling, M-protein can be compared in different samples from the same patient. We have successfully measured M-protein proteotypic peptides extracted from the SPE/IFE gels utilizing SIL peptides and TMT.



Introduction

Serum protein electrophoresis (SPE) is a tool for multiple myeloma (MM) diagnostics used in clinical laboratories.(10, 61) MM is a plasma cell neoplasm characterized by a monoclonal population of plasma cells overproducing a monoclonal immunoglobulin. This monoclonal immunoglobulin, called M-protein, is a biomarker for MM that can be detected and quantified in serum with SPE, followed by immunofixation electrophoresis (IFE), a qualitative assay classifying heavy and light chains of the M-protein.(6, 10, 101) M-protein diagnostics using SPE is an integral part of the laboratory work-up as a screening tool for the identification of MM, and a quantitative biomarker for disease prognostication, to follow the course of the disease, and to monitor response to therapy.(7, 61)

SPE/IFE is a fast and inexpensive diagnostic tool, however, it lacks sensitivity. Novel treatment modalities for MM have led to an increased percentage of patients obtaining stringent complete remission.(59) More sensitive assays capable of measuring minimal residual disease (MRD) are therefore urgently needed. Methods capable to assess MRD, in experimental clinical setting, make use of techniques performed on bone marrow aspirates/biopsies, for example flow cytometry, allele-specific oligonucleotide polymerase chain reaction and next generation sequencing of the immunoglobulin gene rearrangements.(61) Bone marrow aspirates and biopsies are obtained via invasive procedures, moreover they introduce risk of non-representative sampling due to patchy or extramedullary disease.(61, 102) Evaluation of MRD in peripheral blood would represent an attractive minimally invasive alternative to circumvent the above mentioned disadvantages for MRD assessment in bone marrow. Various approaches to monitor the M-protein in serum have been developed utilizing mass spectrometry.(36, 37, 63) Combining the power of genomics and proteomics, we have recently reported a mass spectrometry assay to detect and quantify M-protein in MM patient serum.(37) Utilizing stable isotope labeled (SIL) peptides, mass spectrometry was more than 100 times more sensitive in detection of M-protein compared to current M-protein diagnostics, even in the presence of therapeutic monoclonal antibodies (t-mAbs) daratumumab, ipilimumab and nivolumab, which may interfere with SPE.(103)

On the SPE agarose gel, serum proteins are separated into five visible fractions: albumin, alpha-1, alpha-2, beta and gamma fraction. Detection of M-protein is based on the presence of a well-defined band, usually in the gamma fraction of the electrophoretic gel, called an M-spike. Use of this M-protein band as a starting material for mass spectrometry analysis can eliminate the need for additional serum and immunoglobulin purification from serum for potential automation of MRD analysis in clinical laboratories. We have investigated two different approaches of integrating SPE/IFE with targeted mass spectrometry. We have combined SPE/IFE with the previously developed MS assay for serum and immunoglobulin purified from serum.(37) This assay requires personalized SIL peptides to

measure the M-protein. To circumvent this personalized approach, we have also explored the possibility of tandem mass tag (TMT) labeling of M-protein in MM patient serum with spiked t-mAb. SPE gels are agarose gels supported with a plastic carrier and completely dried after measurement. To measure peptides directly from the SPE gels, M-protein band needs to be cut and the M-protein needs to be extracted for digestion. Before measurement on the mass spectrometer the samples need to be cleaned-up from possible particles coming from the plastic carrier or the dried agarose gel.
We have successfully performed in gel digestion and extracted M-protein specific peptides for targeted analysis on an Orbitrap mass spectrometer using SIL peptides and TMT.

Experimental Section

Biological and other material

All MM patient and control sera, daratumumab and SIL peptides were used as described previously.(37) Briefly, serum of a MM patient is used for which heavy chain Sanger sequencing data is available. All serum samples and clinical data were coded and anonymized as specified in the Dutch code of conduct for biomedical research. Daratumumab was purchased from Janssen Biotech, Leiden, The Netherlands. SIL peptides (Pepscan B.V., Lelystad, The Netherlands), used as internal standards for quantification, were delivered as 5 µM solution in 5% acetonitrile/water and stored at -80°C to preserve integrity until use. Peptide amount and purity were established by amino acid composition analysis, high-performance liquid chromatography (HPLC) combined with MS analysis, by the manufacturer. Peptides, from the patient M-protein and daratumumab, were considered as proteotypic peptides and candidates for stable isotope labeling if they were tryptic peptides with mutations in the amino acid sequence compared to the germline reference sequence. The stable isotope labeled peptides used are shown in Table 1. Figure S-1 in Supporting Information shows the total ion current chromatogram for the peptides selected for daratumumab and the M-protein, and the monitored fragments.

Dilution series preparation for M-protein and daratumumab measurements

To determine the sensitivity of the SPE-MS assay we have prepared series of M-protein dilutions. MM patient serum (16.6 g/L of M-protein defined by densitometry) was diluted into a healthy control serum that did not contain M-protein or daratumumab. In this way, 8 dilutions with 5 fold incremental steps were prepared. M-protein dilution series were prepared in triplicate; two dilution series spiked with daratumumab (at a constant concentration of 1 g/L) and one dilution series without spiked daratumumab. All dilutions and control serum were measured by SPE. Highest concentration M-protein sample was measured in the first and last SPE lane. Figure S-2 in Supporting Information shows the SPE gel scan of the M-protein dilution series spiked with daratumumab. By diluting the MM patient

serum into a control serum we dilute the M-protein, but keep the background proteins at a constant level. That way we can mimic the decreasing tumor load.

To estimate the difference in sensitivity between SPE and IFE as starting materials for mass spectrometry analysis, daratumumab was diluted into a control serum and measured on both SPE and IFE. Two dilutions were prepared: 1 g/L and 0.04 g/L and measured in triplicate; we have measured SPE M-protein band and IFE M-protein band from the IgG lane.

SPE and IFE were performed on a Hydrasys device (Sebia, Evry, France) using reagents from Sebia, according to manufacturer's protocols. Briefly, for SPE 10 µL of serum is applied and allowed to diffuse into the gel at room temperature for 30 seconds; for IFE 10 µL of six times diluted serum is applied and allowed to diffuse into the gel for 60 seconds.

Cutting and digesting SPE and IFE gel bands for proteomic analysis

The gel bands were marked with a ruler and a scalpel, and were cut with scissors. To assure that the bands that are cut from the gels are approximately the same size, ruler was placed directly above and below the band and straight lines with a scalpel were cut in the gel. That straight line was then cut with the scissors, to reduce plastic particles. The gel bands of interest were cut with scissors assuring minimum of plastic around the band. Each band was digested separately in a 1.5 ml Eppendorf tube.

Gel pieces were washed with water, 50% acetonitrile (ACN), 100% ACN and 50% ACN in 100 mM ammonium bicarbonate, then dried in Savant SC210A SpeedVac concentrator (Thermo Fisher, Munich, Germany) for 5 minutes. RapiGest SF (Waters, Milford, MA) 0.1% solution in 50 mM ABC was added to the gel samples and incubated at 37°C for 10 minutes. After drying the gel pieces again in the SpeedVac for 5 minutes, 600 ng of gold-grade trypsin (Promega, Madison, WI) was added to each sample and incubated at 4°C for 5 minutes. Trypsin solution was collected in a separate tube and 50 mM ABC was added to the samples and incubated at 37°C overnight. Tryptic peptides were extracted once with 1% TFA and twice with 0.1% TFA in 50% ACN. All extracts and trypsin solution were combined and completely dried in the SpeedVac. Tryptic peptides from the M-protein and daratumumab were resuspended in 25 µL of 10 fmol/µL SIL peptide solution in 0.1% TFA. Reduction and alkylation were not performed during the sample preparation, because the proteotypic peptides used do not contain cysteine.

Before continuing with the LC-MS analysis all samples were cleaned-up with C18 ZipTips (Millipore, Burlington, MA) in order to preventively remove any particles coming from the plastic gel carrier or dried agarose. ZipTips were used according to manufacturer's protocol. Briefly, after peptide binding to the C18 material, the ZipTip pipette tip is washed with 0.1 % TFA in Milli-Q water, and the peptides are eluted with 0.1% TFA/50% ACN. Figure 1 shows the experimental design for the targeted mass spectrometry workflow using SPE gel as starting material and SIL peptides for quantification.

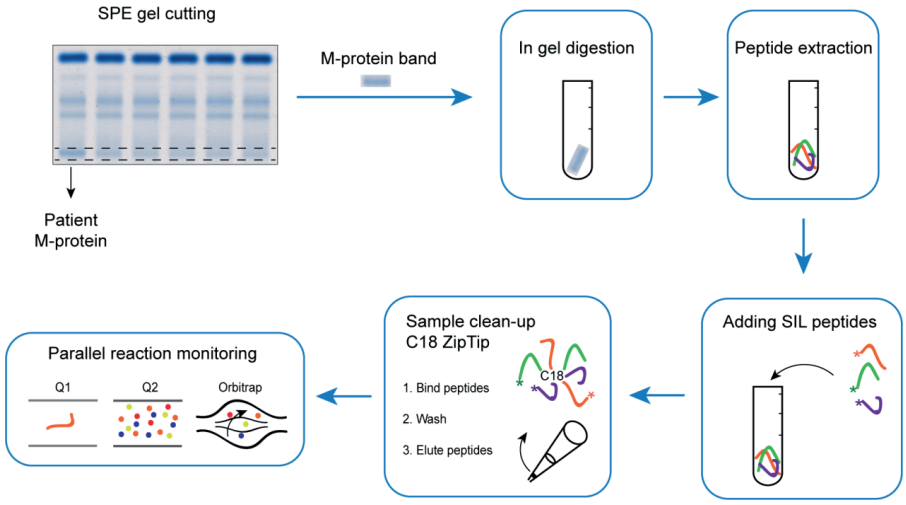


Figure 1. Experimental design for the SPE-MS assay, including serum protein electrophoresis in gel digestion and mass spectrometry measurement. Gel band of interest is cut from the gel and in gel digestion is performed. After peptide extraction, stable isotope labeled (SIL) peptides are added for protein quantification. Samples are cleaned-up with C18 ZipTips and measured with parallel reaction monitoring (PRM) technology.

Liquid chromatography-mass spectrometry (LC-MS) measurements

Liquid chromatography was carried out on a nano-LC system (Ultimate 3000, Thermo Fisher Scientific, Munich, Germany). Samples were separated on a C18 column (C18 PepMap, 75 µm ID × 250 mm, 2 µm particle and 100 Å pore size; Thermo Fisher Scientific, Munich, Germany) and peptides were eluted with the following binary linear gradient of buffer A and B: 4%–38% solvent B in 30 minutes. Solvent A consists of 0.1% aqueous formic acid in water and solvent B consists of 80% acetonitrile and 0.08% aqueous formic acid.

Parallel reaction monitoring (PRM) measurements were performed on Q Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). For electrospray ionization, nano ESI emitters (New Objective, Woburn, MA) were used and a spray voltage of 1.7 kV was applied. For daratumumab and M-protein peptides the instrument was operated in a targeted MS/MS using following parameters: quadrupole isolation width of 4.0 *m/z*, HCD fragmentation, MS/MS AGC target of 500,000 at a maximum injection time of 250 ms, and Orbitrap resolution of 30,000. MS/MS spectra were collected in centroid mode. m/z, charge states, collision energies and fragment types for quantification of daratumumab and M-protein are listed in Table 1.

Table 1. Peptides used for quantification of daratumumab and M-protein.

Protein	Peptide sequence	m/z	z	Collision energy (%)	Extracted fragments
daratumumab	GLEWVSAISGSGGGTYYADSVK	735.3551	3	30	b2, b3, y5, y6, y7
	GLEWVSAISGSGGGTYYADSVK* (90.2)	738.0265			
M-protein	GLEWVSYISSGGGSTYYADSVK	1163.5473	2	21	b5, b8, y12, y13, y14, y15
	GLEWVSYISSGGGSTYYADSVK* (90.3)	1167.5544			

*stable isotope labeled amino acid, (percentage purity of the stable isotope labeled peptide)

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD016325.

Data processing

Signals were integrated using Skyline.(70) We have selected a maximum of six transitions with highest intensities for each peptide. The concentration of each peptide was calculated from the peak area ratio between the endogenous and the SIL peptides. Peak areas were calculated as the sum of all selected transitions. Calculation of M-protein concentration from PRM data is shown in Supporting Information in Figure S-3. Limit of detection (LOD) for the M-protein was defined as (3.3 x SD)/slope, and lower limit of quantification (LLoQ) as 3 x LOD, according to ICH guidelines (http://www.ich.org); where SD was calculated for control serum without M-protein and with low concentration of M-protein (two highest dilutions). Recovery (%) was calculated for both M-protein and daratumumab by dividing measured concentration of M-protein with expected concentration of M-protein. Expected concentration is the M-protein concentration in the serum that is loaded on the SPE.

Tandem Mass Tag (TMT) labeling

For TMT labeling SPE gels and the M-protein dilution series were prepared as described for the SIL labeling approach. The same MM patient serum was diluted into a control serum with 1 g/L of ipilimumab (Brystol-Myers Squibb, Utrecht, The Netherlands). TMTsixplex Isobaric Label Reagent Set was purchased from Thermo Fisher Scientific. For samples labeled with TMT, digestion of the gel bands was performed without Rapigest, ABC was exchanged for triethylammonium bicarbonate (TEAB) buffer and TFA was neutralized with TEAB before adding the TMT labels to the samples. TMT labeling was performed according to manufacturer's protocols. Two samples were labeled with TMT and they will be referred to as M-protein high concentration sample (3.3 g/L of M-protein, 1 g/L of ipilimumab) and M-protein low concentration sample (5.3 mg/L of M-protein, 1 g/L of ipilimumab). M-protein high concentration sample was labeled with TMT^6-127 label (127.1248 monoisotopic reporter mass), and M-protein low concentration sample was labeled with TMT^6-126 (126.1277 monoisotopic reporter mass). Figure 2 shows the experimental design for the mass spectrometry workflow using TMT for

labeling the M-protein and ipilimumab proteotypic peptides. After labeling, samples were mixed and targeted mass spectrometry analysis was performed.

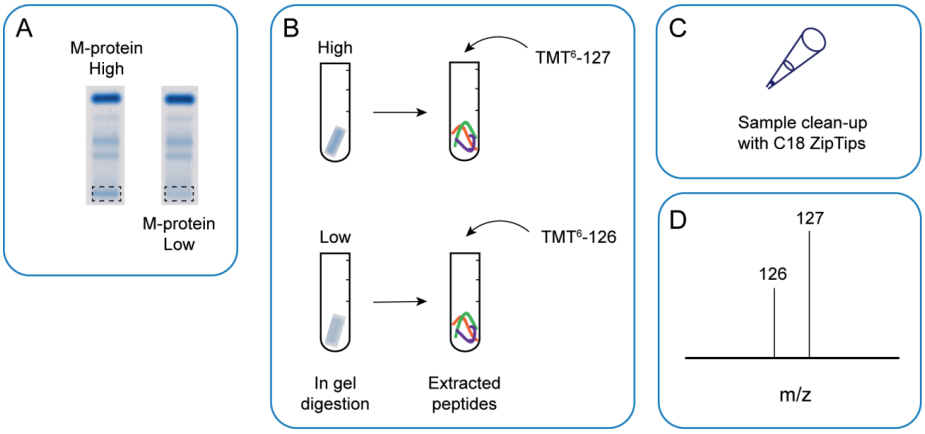


Figure 2. Experimental design for tandem mass tag (TMT) labeling of peptides extracted from serum protein electrophoretic gel bands. Two samples with high and low M-protein concentration (A) are labeled with two different TMT labels (B), samples are cleaned-up using C18 ZipTips (C), and measured on the mass spectrometer (D). Concentration of the low concentration M-protein sample can now be calculated from the ratio between the two reporter ions and known concentration of the high (baseline) concentration serum sample.

Mass spectrometry measurements were performed on Orbitrap Fusion (Thermo Fisher Scientific). For ipilimumab and M-protein peptides the instrument was operated in a targeted MS/MS using following parameters: quadrupole isolation width of 1.4 *m/z*, HCD fragmentation, MS/MS AGC target of 50,000 at a maximum injection time of 246 ms, and Orbitrap resolution of 120,000. MS/MS spectra were collected in centroid mode. m/z, charge states and collision energies of ipilimumab and M-protein are listed in Table 2.

Table 2. Sequences and masses of peptides labeled with TMT, at both the N-terminal amine and at the amine of lysine.

Protein	Peptide sequence	m/z	z	Collision energy (%)
ipilimumab	T^{TMT}GWLGPFDYWGQGTLVTVSSASTK^{TMT}	1006.1989	3	38
M-protein	G^{TMT}LEWVSYISSGGGSTYYADSVK^{TMT}	1392.7103	2	38

Results

Removing contamination/particles coming from the SPE gel pieces

C18 ZipTips were used as a filter to remove any particles or contamination coming from plastic gel carrier/dried agarose that might interfere with the assay. The clean-up, using C18 ZipTips, was performed successfully for all samples, and they were separated and analyzed on the LC-MS systems without excluding any sample.

Sensitivity of the SPE-MS assay

Proteotypic peptides for the M-protein and daratumumab were successfully identified and quantified. Addition of daratumumab shows that two different monoclonal antibodies can be identified from the same SPE gel band without interference.

LOD and LLoQ were determined for the peptide GLEWVSYISSGGGSTYYADSVK of the M-protein. LOD is 2.4 mg/L and LLoQ is 7.3 mg/L. Dilution series of the M-protein was measured in triplicate as shown in Figure 3. Measurements were linear in the measured range above LLoQ (r^2 = 0.95). Table S-1 in Supporting Information shows coefficient of variation (CV) and standard deviation (SD) values for daratumumab and M-protein. Recovery (with standard deviation) from the serum, that was measured on the SPE gel, was calculated for both the M-protein and daratumumab, relative to the amount of serum loaded on the gel. Recovery for M-protein is 1.34% (0.42) and it was calculated as an average of first 5 dilutions; which were above the LLoQ. For daratumumab recovery is 0.87% (0.18) and it was calculated as an average of all 8 dilutions with spiked daratumumab. The volume of serum that is applied on the SPE sample applicator is 10 µL, however because of the way the SPE is performed, it has been reported that just a small fraction of the sample is actually introduced into the gel.(104) If just a fraction of serum enters the gel, it is evident that the amount that will be measured by mass spectrometry will be proportionally lower. We have used 10 µL, as the volume of serum, to calculate the concentration of the M-protein in serum (Figure S-3.), meaning that the recovery we calculate includes the loss of serum on the SPE/IFE applicator, loss during the digestion and loss during C18 ZipTip clean-up. It is a recovery for the integrated SPE-MS workflow and as a result the numbers are relatively low.

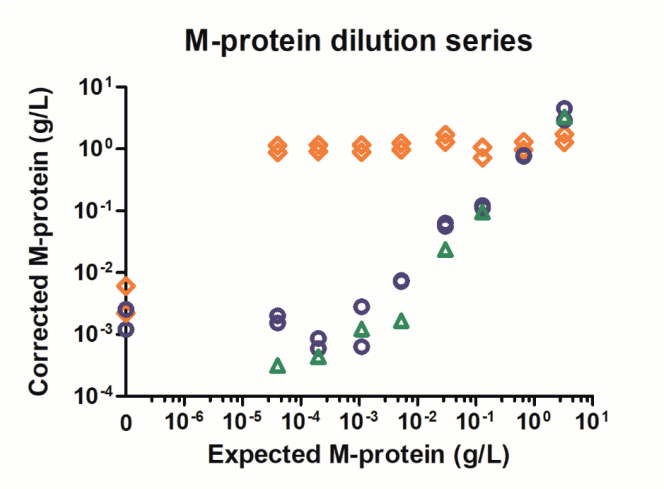


Figure 3. Dilution series of the M-protein in a control serum matrix with and without spiked daratumumab (1 g/L). Blue circles represent data points for the M-protein from the dilution series in the presence of spiked daratumumab, orange diamonds represent data points for daratumumab, and green triangles represent data points for the M-protein from the dilution series without spiked daratumumab. Measured concentration of the M-protein in each dilution, corrected for the recovery calculated for the highest concentration M-protein sample (3.3 g/L of the M-protein), is shown on the y-axis. Data points for blank measurements (control serum) are plotted at 0. Linear line was fit ($y = 1.0841x + 0.0073$, $r^2 = 0.95$) based on the data points above the LLoQ for the M-protein (top four).

SPE and IFE as starting materials for the MS assay

To estimate the difference in sensitivity between SPE and IFE as starting materials for mass spectrometry measurements, two dilutions of daratumumab (1 g/L and 0.04 g/L) in control serum were measured on both SPE and IFE gels. As showed in Figure 4, maximum heights of the peaks corresponding to the endogenous peptide targeted for daratumumab (GLEWVSAISGSGGGTYYADSVK) are higher in SPE than IFE (IgG lane) samples for both dilutions. Average peak maximum is 5 times higher in SPE than in IFE for the lower concentration (0.04 g/L), and 7 times higher in SPE than in IFE for the higher concentration (1 g/L). Maximum heights of the peaks for the SIL peptide, which was added to all samples in the same concentration (10 fmol/µL), were measured as a control. Peaks for the SIL peptide do not show this difference in maximum peak height.

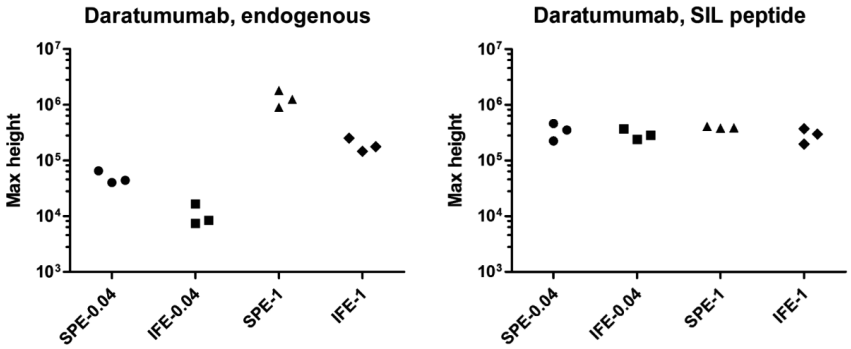


Figure 4. Comparison of SPE and IFE as starting materials for mass spectrometry. Two dilutions of daratumumab (0.04 g/L and 1 g/L) in control serum were measured in triplicate on SPE and IFE (IgG lane). Maximum heights of the peaks corresponding to daratumumab endogenous peptide are higher in SPE than IFE (IgG lane) samples for both dilutions. Maximum heights of the peaks for the stable isotope labeled (SIL) peptide, which was added in the same concentration in all samples, were measured as a control and they do not show this difference in maximum peak height.

TMT labeling of MM patient serum

The targeted MS assay using SIL peptides requires synthesis and stable isotope labeling of proteotypic M-protein peptides for every new patient. To explore an alternative workflow that circumvents the need for the personalized labeling approach, MM patient serum with added ipilimumab was labeled with tandem mass tags. Two samples with different M-protein concentrations, and the same ipilimumab concentration (1 g/L), were labeled with two different TMT labels, TMT^6-127 and TMT^6-126. After labeling the M-protein high concentration sample (3.3 g/L of M-protein) and M-protein low concentration sample (5.3 mg/L of M-protein), samples were mixed and measured. Both reporter ions can be observed in the MS^2 spectra, as shown in Figure 5. We have labeled two M-protein samples as the rationale behind the high and the low concentration M-protein sample is that the baseline serum sample with high tumor load and high M-protein concentration is quantified by SPE in routine clinical laboratory testing. The concentration of the M-protein is then known, therefore the baseline sample can be used as a reference for follow-up samples. Samples with an M-protein concentration lower than the limit of quantification of the SPE are ideally further investigated with a more sensitive method. Concentration of the low M-protein sample can now be calculated from the ratio between the two reporter ions and known concentration of the baseline serum sample. Both samples, high and low concentration of the M-protein, were successfully labeled and ipilimumab is distinguished from the M-protein (Figure 5.).

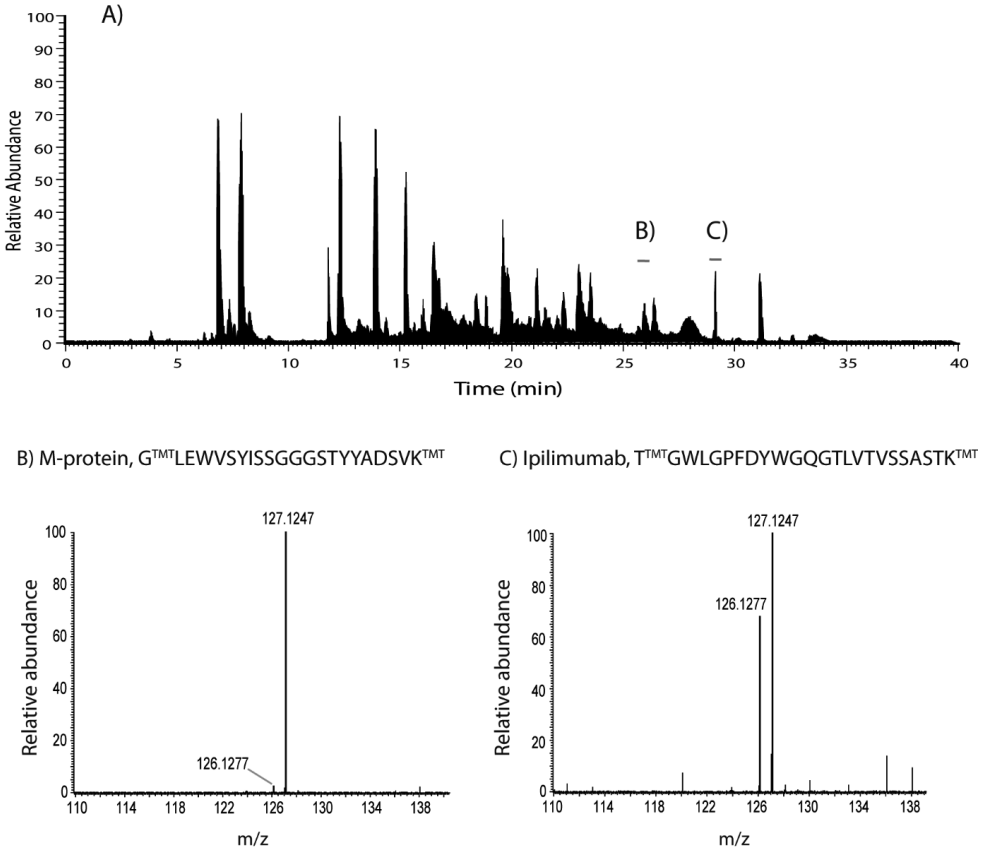


Figure 5. Tandem mass tag (TMT) labeling results for the mixture of two samples with different M-protein concentrations, with added 1 g/L of ipilimumab. Green lines in the MS^2 chromatogram (A) indicate the integrated area to generate MS^2 spectra for the M-protein (B) and ipilimumab (C). High concentration M-protein sample (3.3 g/L of M-protein) was labeled with TMT^6-127 and low concentration M-protein sample (5.3 mg/L of M-protein) was labeled with TMT^6-126. Both reporter ions are detected in the MS^2 spectra.

Discussion

The SPE-MS targeted M-protein assay, with LOD of 2.4 mg/L exceeds the LOD of SPE (LOD = 200 – 400 mg/L).(105) SPE and IFE are not sensitive enough to give information about the depth of response in MM patients, however, they are fast and inexpensive tools for MM diagnosis and patient screening. Separation of monoclonal antibodies in SPE in combination with Orbitrap mass spectrometry enable sensitive and targeted measurement of the M-protein. The SPE-MS assay can therefore be used for MM patient follow-up, when a more sensitive method than electrophoresis is needed to determine the disease load, for example during MRD. Previously developed targeted assay for M-protein

measurement in serum and immunoglobulin purified from serum had an LOD of 2.1 mg/L and 0.2 mg/L, respectively, for M-protein from the same patient as described in this study.(37) The MS assay performance that would be required to detect and quantify the M-protein during MRD is still unknown. More knowledge about the M-protein concentrations during MRD will help to assess whether further improvement in assay sensitivity is necessary. The M-protein concentration and the clinical benefit of measuring and acting on the M-protein during MRD need further characterization in larger patient groups.

With implementation of the SPE-MS assay into a clinical laboratory in mind, it is good to consider characteristics of the proteotypic peptide candidates and SIL peptide standards. As the MS assay is personalized, the LOD could be different for different patients, as every patient will have different peptides acting as surrogates for the M-protein. Additionally, peptide candidates from the same patient could have different sensitivities. Sensitivity assessment can aid in choosing the best peptide candidates. The MS^2 signal of a peptide contains different transitions with a characteristic intensity distribution, comparison with reference spectra allows for a good assessment of signal quality obtained at lower concentration levels. Batch to batch purity of a SIL peptide may differ, and can be taken into account for M-protein quantification, especially when different peptide batches are anticipated. When selecting proteotypic peptides, it is also good to pay attention to several amino acids that can have certain modifications, for example oxidation of methionine (M) and tryptophan (W).(106) Especially methionine oxidation was prevalent in comparable gel samples analyzed by shotgun LC-MS, and only a limited amount of oxidation of tryptophan and histidine residues. When the use of methionine containing peptides cannot be avoided, the complete oxidation of methionine with e.g. hydrogen peroxide can be a useful sample preparation strategy to mitigate the analytical consequences of sample oxidation.

Recovery of the M-protein and daratumumab from serum was calculated by dividing concentration of M-protein measured on the mass spectrometer with the M-protein concentration in the serum that is loaded on the SPE. The recovery that was found raises the question on how to compensate for recovery in a quantitative test, as the internal standard peptide can only be added after electrophoresis and digestion. MS quantification of an M-protein from an SPE gel can be performed in several ways. Recovery can be calculated for a baseline, high abundant, M-protein sample for which M-protein concentration can be determined with both SPE (densitometry) and the SPE-MS assay. That recovery can be used to determine concentration of the unknown, follow-up, sample, if the SPE and MS are performed in the same way. The baseline patient sample is used as a reference standard for samples with M-protein concentration below the sensitivity of the SPE, that is, when the M-protein band is not visible on the SPE gel. The M-protein band of the follow-up sample can be expected and

cut at the same position in the SPE lane as the M-protein band of the baseline patient sample. The potential risk of technical errors when preparing the SPE gel has to be taken into account and non-consistent band migration avoided. Inclusion of a known reference sample could provide validation that the expected migration pattern was indeed reproduced. Alternatively, a compound of known concentration can be added to the serum before SPE analysis, for example a monoclonal antibody, however this is not feasible for the analysis of archived gel samples. In our case, daratumumab was spiked in all samples measured, except control serum matrix. To assure straight cutting of the gel bands, highest concentration M-protein sample was measured in the first and the last SPE lane. We have calculated the linearity of the SPE-MS assay and have obtained a high r^2 value. The SPE-MS assay would not be applicable for patients with no detectable band on the SPE gel for the baseline sample.

In gel digestion can be performed on both SPE and IFE gels bands. We have used both SPE and IFE as input materials for integrated MS assay. Even though IFE is a more sensitive method in the routine diagnostics(101), SPE is a better starting material for MS measurement. The reduction in the maximum peak height in the IFE samples (5 times for the lower concentration sample and 7 times for the higher concentration M-protein sample) is fitting with the serum dilution that is performed before IFE (6 times diluted serum). As both gel methods use distinct procedures for the introduction of serum into the gel, this difference in sensitivity may simply be a consequence of the increased amount of material in the SPE gel.

The SPE-MS assay measures the M-protein in a targeted way and can therefore aid in analysis of ambiguous bands and circumvent diagnostic problems of SPE. For example interference of t-mAbs, cases of biclonal gammopathy or M-protein band in the beta region of the SPE lane.

Quantification of an M-protein that migrates in the beta region is compromised by densitometry because of the presence of other serum proteins. The SPE-MS targeted assay measures peptides specific for the patient M-protein, therefore circumventing the interference from other peptides in the sample.

T-mAbs can interfere with M-protein diagnostics, because of co-migration with the M-protein on the SPE gels.(103) T-mAbs do not interfere with the MS assay. By adding appropriate reference peptides, both M-protein and the t-mAbs can be measured in a single run. Similarly, using targeted MS, it is possible to analyze the immunoglobulin class of the M-protein by adding appropriate reference peptides for different immunoglobulin varieties to the assay.(29)

MM patients usually have one plasma cell clone producing one monoclonal M-protein consisting of a specific combination of a heavy and a light chain. However, sometimes more than one monoclonal protein can be identified. In the case of biclonal gammopathy, double M-protein bands can be

observed on the SPE gel. These two M-proteins have different heavy and/or light chains.(107) The SPE-MS approach can be of value in analyzing such bands.

SPE could be streamlined with mass spectrometry and a fully automated system could be developed, which would make the assay more accessible in a clinical laboratory setting. The SPE-MS assay can also be used for analysis of archived SPE gels. We have performed experiments with SPE gels that are more than 10 years old, and, by cutting and digesting the albumin band, we were successful in measuring albumin peptides on the mass spectrometer. SPE gels can be archived under ambient conditions and they are a great source for retrospective M-protein analysis. For example, retrospective gel samples can be used to investigate relationship between serum concentration of M-protein, the administration of t-mAbs and disease response.

The SIL peptide approach enables absolute quantification of the endogenous peptide. The peptide and therefore the protein concentration can be calculated from the peak area ratio between the endogenous and the SIL peptide. In addition, the SIL peptide corrects for potential loss of the endogenous peptide due to, for example, adsorption on the surface of the vial. However, SIL peptides are personalized reagents and have to be synthesized for every patient, which may increase the cost of the method, additionally, it may increase the turnaround time of the method set up. TMT is a non-personalized label that can be used for all MM patients, potentially lowering the turnaround time and lowering the cost of the method. Follow-up patient samples, during the disease course, can be measured and M-protein concentrations compared. The protein quantification, with the TMT approach, is different than when using SIL peptides. To quantify M-protein, using the TMT approach, a reference sample is needed, that is a sample with a known concentration of the M-protein. M-protein concentration is then calculated from the ratio between the reporter ions of the reference sample and the follow-up sample of the unknown concentration.

For both the SIL and TMT labeling approach, having the information about the M-protein sequence is helpful, as it guarantees that the correct MS peak is being selected. One bone marrow sample is necessary to retrieve the M-protein sequence. For clinical purposes and routine diagnostics this will make the measurements more robust and reliable.

Comparison of the SIL and TMT approaches, for example of applicability, cost and turnaround time for measuring the M-protein, has not yet been performed in a clinical setting. The method of choice will be influenced by shortcomings and advantages reported in a clinical laboratory. A protocol can be envisioned where MM patient samples are measured by SPE and IFE, being the routine M-protein diagnostic techniques appropriate for measuring high M-protein concentrations; when the sensitivity of SPE and IFE is not enough for M-protein detection and the results are ambiguous, mass spectrometry assay can be used for sensitive M-protein detection during minimal residual disease.

Conclusion

We have successfully digested M-protein bands from the serum protein electrophoretic gels, extracted M-protein proteotypic peptides and removed any contamination using C18 ZipTip pipette tips. Targeted mass spectrometry analysis was performed and patient M-protein was quantified using stable isotope labeled peptides. Sensitivity of SPE-MS approach exceeds the sensitivity of serum protein electrophoresis by two orders of magnitude.

We have also explored a non-personalized labeling approach using tandem mass tags and we have succeeded to label peptides, extracted from the SPE gels, with tandem mass tags in order to compare follow-up samples from the same patient with different M-protein concentrations. This approach has the potential to allow a personalized test without using personalized reagents.

Acknowledgements

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Chapter 7

General discussion

Summary

Samenvatting

General discussion

Multiple myeloma (MM), a plasma cell malignancy arising in the bone marrow (129), accounts for 10% of all hematologic malignancies.(130) According to the World Health Organization (WHO)'s International Agency for Research on Cancer (http://globocan.iarc.fr/), approximately 48 000 people are diagnosed with MM annually in Europe.

In the past decade, the treatment of multiple myeloma has advanced from conventional chemotherapy drugs to novel agents including immunomodulatory drugs (thalidomide, lenalidomide, and pomalidomide), proteasome inhibitors (bortezomib) and therapeutic monoclonal antibodies (daratumumab) with limited toxicity.(131) Novel agents and therapeutic combinations have led to more MM patients achieving complete remission.(132) Advancements in patient treatment and availability of new assays for myeloma detection and monitoring led to redefinition of myeloma response criteria. Definition of stringent complete remission was included to multiple myeloma response criteria by adding serum free light chain analysis, to routine serum and urine electrophoretic analysis of monoclonal proteins and bone marrow assessment.(61) However, even patients with such deep responses eventually relapse indicating that the cancer is not absent, it is just not detectable with current techniques. Thus innovative sensitive technologies are needed to detect minimal residual disease (MRD) and help guide patient treatment.

Current technologies for MRD testing, such as flow cytometry and next-generation sequencing, are bone marrow-based. Bone marrow sampling could introduce a risk of non-representative sampling due to patchy or extramedullary disease.(85) Additionally, invasiveness of repeated bone marrow punctures reduces the quality of life of MM patients.

This study aimed to develop a sensitive mass spectrometry (MS) assay to measure MRD in MM patients. A highly sensitive, blood-based MS assay for MRD detection would provide better quality of life and would support clinical decision-making. The possibility of a blood-based assay would allow frequent disease monitoring and more sensitivity would mean greater ability to adjust patient treatment.

The developed MS assay measures patient-specific peptides from the M-protein, the product of malignant plasma cells. Specificity of the MS assay relies on the selection of patient-specific M-protein peptides, thereby assuring measurement of only M-protein and not any other immunoglobulin from the polyclonal background. Personalized nature of the MS assay is a limitation as for each patient the M-protein sequence has to be known and the patient-specific M-protein peptides have to be selected. To select patient-specific peptides from sequencing data, a bone marrow sample at diagnosis is needed. However, after the initial set-up, the assay is ready to use and the turnaround time drops significantly, with the advantage of high sensitivity.

In **Chapter 3**, patient-specific M-protein peptides were selected for 23 MM patients. Criteria used for peptide selection resulted in patient-specific peptides from heavy and light M-protein chains. Peptide peaks were detected in corresponding MM patient sera and not in the control samples showing that successful peptide selection leads to high specificity. Patient-specific M-protein peptides were successfully selected from DNA sequencing (**Chapters 2** and **4**), RNA sequencing (**Chapters 3** and **6**) or *de novo* proteomic sequencing (**Chapter 5**). Approximately 2% of patients with multiple myeloma do not secret an M-protein.(130) The MS assay and the electrophoretic techniques are not applicable for patients suffering from non-secretory disease. For these patients bone-marrow-based techniques are essential for detecting malignant plasma cells.

Multiple monoclonal immunoglobulins can be detected and quantified simultaneously using the MS assay. The use of therapeutic monoclonal antibodies can cause interference with the conventional electrophoretic techniques used for M-protein diagnostics.(103) In **Chapters 2** and **4**, M-protein and three different therapeutic monoclonal antibodies were measured using the MS assay in serum and excised serum protein electrophoretic (SPE) bands, respectively. The therapeutic antibodies did not cause interference in the MS assay. In addition, the M-protein and the therapeutics can be quantified in a single MS experiment; even small amounts of therapeutic monoclonal antibodies (down to 1 mg/L) can be measured in blood and other body fluids. In **Chapter 6**, we have quantified daratumumab in serum and cerebrospinal fluid of a myeloma patient with leptomeningeal involvement in order to investigate the ability of daratumumab to cross the blood-brain barrier. Therapeutic drug monitoring of monoclonal antibody therapy using the MS assay could guide more effective dosing in cancer and inflammatory diseases.(133)

The MS assay is a highly sensitive assay, more than 100 times more sensitive than conventional M-protein diagnostics, shown in **Chapters 2**, **3** and **4**. Superior sensitivity over conventional M-protein diagnostics, in combination with blood-based sampling is the main strength of the MS assay. Blood sampling allows frequent M-protein monitoring and investigation of the kinetics of M-protein. The MS assay can detect an increase in M-protein levels more than 100 days earlier than realized with conventional electrophoresis (**Chapter 5**). MS can detect increasing M-protein levels after stopping treatment and even during maintenance therapy. Thus sensitive and longitudinal M-protein monitoring could influence treatment decisions and aid in early detection of relapse. The MS assay provides M-protein concentration data that gives information about the patient MRD status complementary to plasma cell data acquired by flow cytometry (**Chapter 3**). MS has the potential to reduce the need for bone marrow sampling and monitor the minimal residual disease in a minimally invasive way.

Absolute quantification of the M-protein is performed by comparing the patient-specific M-protein peptide to a stable isotope labeled peptide, used as an internal standard. Assays used in a clinical setting have to be standardized across institutions and clinical trials. An internal standard offers the possibility for standardization of the assay across clinical laboratories. Alternatively to personalized internal standards, peptides can be labeled with tandem mass tags (TMT). In **Chapter 4**, TMT was used to measure the M-protein and compare the M-protein levels in multiple samples from a multiple myeloma patient.

Serum, serum enriched for immunoglobulins and bands taken from either SPE or immunofixation electrophoretic (IFE) gels were investigated as starting materials for the targeted MS assay in **Chapters 2** and **4**. The MS serum assay can be performed with small sample volumes (approximately 10 microliters of serum), whereas the combined SPE-MS assay requires only an M-protein band. Molecular and cellular methods for minimal residual disease monitoring, for example flow cytometry, require bone marrow punctures which are highly invasive and the samples need to be processed fresh. Serum samples are minimally invasive and can be easily transported. In addition, gel samples are stable at room temperature for long periods of time (decades) making it possible to retrospectively select patient samples from clinical laboratory archives (**Chapter 5**). Prospective patient samples may take years to collect. Thus SPE gels, retrospectively selected from clinical trials or clinical laboratory archives, are of great value to show the sensitivity of the MS assay for measuring the M-protein during years of patient disease course. Using mass spectrometry, changes in M-protein levels can be seen throughout the patient disease courses. The MS assay can monitor patients with subtle differences in M-protein levels and also patients in apparent remission.

Future perspectives

The MS assay for minimal residual disease monitoring is a minimally invasive and highly sensitive assay that enables longitudinal follow-up of multiple myeloma patients. Accurate quantification of the M-protein using TMT, and alternative labeling approaches, could be explored to circumvent the use of patient-specific internal standards and reduce the turnaround time of the MS assay.

MS technology is becoming more accessible at economically viable prices and clinical applications of mass spectrometry continue to expand.(134) To implement the MS assay into routine clinical practice certain steps need to be taken for method verification that are specific for MS technology. Biological variability, inter-assay imprecision and robust threshold for MRD-negativity have to be defined. Streamlining and automation of sample processing would make the assay more clinical laboratory-friendly. Education and training of laboratory personnel how to execute the assay would need to be

done in order to increase awareness of the benefits of the MS assay and to implement the assay in clinical practice.

The MS assay should most ideally be included in new clinical trials in order to investigate the clinical value of MS-MRD-guided treatment decisions. One could hypothesize that MS-MRD-positivity calls for escalation of therapy or alternative therapies, while achieving MS-MRD-negativity, i.e. clearance of the M-protein, allows for de-escalation of therapy. However, these hypotheses have to be explored in large clinical trials. Data from clinical trials in which MS is included for MRD evaluation are needed to investigate the clinical value of the MS assay.

Many patients, who achieve complete response, are on long-term maintenance. Phase 3 clinical trials have shown that maintenance therapy prolongs progression-free survival.(135) With maintenance therapy more patients are in complete remission, compared with the patients who are not on maintenance. Question remains how long does a patient needs to be on maintenance therapy if they are MRD-positive or MRD-negative. Inclusion of the sensitive MS assay to clinical trials could provide an answer to this question. Data from clinical trials will add valuable information about the patient MS-MRD status and could aid in guiding clinical decisions and answering if maintenance treatment could be stopped for a patient with sustained MS-MRD-negativity for a certain amount of time.

The perspective is to perform the validation, accelerate market uptake, and to use the MS technology in a clinical laboratory setting.

Summary

The research described in this thesis focuses on minimal residual disease monitoring in multiple myeloma using mass spectrometry. Sensitive determination of minimal residual disease in patients with multiple myeloma is becoming more important given the increase in the number of patients who achieve complete remission and nevertheless relapse. Current techniques for minimal residual disease monitoring require invasive bone marrow punctures and are therefore not suitable for frequent patient follow-up. Using mass spectrometry, M-protein, the product of malignant plasma cells, can be detected and quantified in blood-based samples of multiple myeloma patients.

In **Chapter 2**, a serum-based targeted mass spectrometry assay was developed in order to measure low levels of M-protein even in the presence of therapeutic monoclonal antibodies. The mass spectrometry assay method measures patient specific M-protein peptides and quantifies them using a stable isotope labeled internal standard. Patient-specific M-protein peptides are selected from the DNA or RNA sequence information. The developed mass spectrometry assay can circumvent repeated bone marrow sampling and enables simultaneous quantification of M-protein and therapeutic monoclonal antibodies. The mass spectrometry assay is more than 100 times more sensitive than routine electrophoretic M-protein diagnostics.

The targeted mass spectrometry assay was performed on 25 follow-up samples from 23 multiple myeloma patients. M-protein was detected in all follow-up serum samples measured using mass spectrometry, while only 5 patients were minimal residual disease positive by flow cytometry. Data presented in **Chapter 3** show that the mass spectrometry assay provides information about the M-protein levels and the patient minimal residual disease that can complement plasma cell data acquired with flow cytometry.

In **Chapter 4**, M-protein bands from serum protein electrophoretic gels were analyzed using the mass spectrometry assay. Serum protein electrophoresis is a fast and inexpensive test used for M-protein diagnostics, however it is not very sensitive for monitoring minimal residual disease. M-protein gel bands can be used as the starting material for the mass spectrometry assay, resulting in the same sensitivity for detecting the M-protein as the mass spectrometry assay starting from serum. Patient-specific M-protein peptides and peptides from the therapeutic monoclonal antibodies were successfully extracted and measured from the serum protein electrophoretic gels.

In **Chapter 5**, serum protein electrophoretic gels from ten patients were analyzed. To circumvent the need for bone marrow sample and DNA or RNA sequencing, proteomic *de novo* sequencing was performed. In all ten patients, at least one patient-specific M-protein peptide was identified using *de novo* sequencing and the sensitivity of the mass spectrometry assay allowed longitudinal monitoring of the patient M-protein.

Chapter 6 describes a multiple myeloma patient with leptomeningeal involvement, who was receiving daratumumab, a therapeutic monoclonal antibody, for maintenance treatment. Using the mass spectrometry assay, M-protein and daratumumab were measured in both serum and cerebrospinal fluid of this patient in order to estimate the penetration of daratumumab across the blood-brain barrier. The analysis showed that the penetration of systemic daratumumab into the central nervous system is limited, which may affect the therapeutic value for the treatment of leptomeningeal myeloma.

In conclusion, the MS assay is a minimally invasive and highly sensitive assay that enables early detection of cancer and allows frequent longitudinal follow-up of multiple myeloma patients. The assay has the potential to better support the hematologist in patient treatment strategy that does not require the patient to undergo repetitive bone marrow punctures.

Samenvatting

Het onderzoek beschreven in dit proefschrift is gericht op het monitoren van minimale restziekte in patiënten met multipel myeloom waarbij gebruik wordt gemakt van massaspectrometrie. Het gevoelig kunnen vaststellen van minimale restziekte in patiënten met multipel myeloom wordt steeds belangrijker gezien de toename van het aantal patiënten die complete remissie bereiken en desondanks een relapse ontwikkelen. Het vaststellen van minimale restziekte wordt tot op heden vastgesteld met methoden die gebruik maken van beenmerg puncties. Hoewel deze methoden grote klinische waarde hebben, zijn ze vanwege het invasieve karakter ongeschikt voor het dynamisch volgen van de ziekte activiteit. Met massaspectrometrie kan M-proteïne, het antilichaam product dat wordt uitgescheiden door maligne plasma cellen, gedetecteerd en gekwantificeerd worden in het bloed.

In **Hoofdstuk 2**, wordt een gerichte massaspectrometrie methode ontwikkeld die in serum lage concentraties van het M-proteïne kan meten in aanwezigheid van therapeutische monoclonale antilichamen. De massaspectrometrie methode meet patiënt specifieke M-proteïne peptiden en kwantificeert deze met behulp van een stabiele isotoop gelabelde interne standaard. Patiënt specifieke M-proteïne peptiden werden geselecteerd op basis van de DNA of RNA sequentie van het M-proteïne. Het herhaaldelijk afnemen van beenmergpuncties wordt overbodig met de nieuwe ontwikkelde massaspectrometrie methode en daarnaast is het ook mogelijk om het M-proteïne en de therapeutische monoclonale antilichamen tegelijkertijd te kwantificeren. De massaspectrometrie methode is ook nog eens meer dan 100 keer zo gevoelig als de huidige standaard elektroforese diagnostiek.

Met de gerichte massaspectrometrie methode zijn 25 follow-up samples van 23 multipel myeloom patiënten gemeten. In alle follow-up samples is met de massaspectrometrie methode M-proteïne gedetecteerd, terwijl slechts 5 patiënten positief bevonden werden voor minimale restziekte op basis van flowcytometrie metingen. De resultaten, beschreven in **Hoofdstuk 3,** geven aan dat de massaspectrometrie methode informatie geeft over de M-proteïne concentratie en de status van de minimale restziekte. Deze informatie kan een toegevoegde waarde hebben op de verkregen informatie met flowcytometrie van plasma cellen.

In **Hoofdstuk 4**, wordt beschreven hoe ook M-proteïne bandjes geïsoleerd van serum eiwitelektroforese gelen gemeten kunnen worden met massaspectrometrie. Serum eiwitelektroforese is een snelle en goedkope test die standaard gebruikt wordt voor de detectie van M-proteïne, maar deze test is niet gevoelig genoeg om minimale restziekte te meten. Behalve serum kunnen ook gel-bandjes van het M-proteïne gebruikt worden als start materiaal voor de massaspectrometrie methode. Met zowel gel-bandjes als serum kan eenzelfde gevoeligheid behaald

worden met massaspectrometrie. Hieruit blijkt dat patiënt specifieke M-proteïne peptide en therapeutische monoclonale antilichamen peptiden succesvol geëxtraheerd en gemeten kunnen worden uit de serum eiwitelektroforese bandjes van het M-proteïne.

In **Hoofdstuk 5**, wordt de analyse van M-proteïne uit serum eiwitelektroforese gels van tien patiënten beschreven. DNA- en RNA-sequencing op beenmerg werd voorkomen door het toepassen van *de novo*-sequencing van het proteoom in de gels. In alle tien patiënten werd tenminste één patiënt specifieke M-proteïne peptide geïdentificeerd met *de novo*-sequencing. De gevoelige massaspectrometrie methode maakte het mogelijk om de patiënt specifieke M-proteïne in de loop van de ziekte te kunnen meten.

In **Hoofdstuk 6** wordt een leptomeningeale multipel myeloom patiënt beschreven die als onderhoudsbehandeling daratumumab krijgt toegediend, een monoclonale therapeutisch antilichaam. Gebruikt makend van de massaspectrometrie methode werden zowel het M-proteïne als daratumumab gemeten in serum en in hersenvocht van deze patiënt om te onderzoeken of daratumumab vanuit het bloed door de bloed-hersenbarrière naar het centrale zenuwstelsel kan gaan. Uit de metingen bleek dat daratumumab slechts beperkt door de bloed-hersenbarrière heen gaat, hetgeen een effect kan hebben op de behandeling van leptomeningeale multipel myeloom.

De massaspectrometrie methode is erg gevoelig en daarbij ook nog eens minimaal invasief wanneer gebruik gemaakt wordt van bloed en elektroforese gelen, waarbij de patiënt door de tijd heen gevolgd kan worden. De meerwaarde van de MS analyse in bloed ligt vooral in het frequenter kunnen monitoren van minimale restziekte dat het vervolgen van therapie-effectiviteit, vroege detectie van een relapse en het beoordelen van extramedullaire ziekteactiviteit vergemakkelijkt. De assay heeft de potentie om de hematoloog beter te ondersteunen bij behandelingsstrategie waarbij de patiënt geen frequente beenmerg puncties hoeft te ondergaan.

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Appendices

List of publications

PhD portfolio

Biography

List of publications

Zajec M, Jacobs JFM, Groenen PJTA, de Kat Angelino CM, Stingl C, Luider TM, De Rijke YB, VanDuijn MM. Development of a Targeted Mass-Spectrometry Serum Assay To Quantify M-Protein in the Presence of Therapeutic Monoclonal Antibodies. J Proteome Res. 2018;17(3):1326-33.

Snijders MLH, **Zajec M**, Walter LAJ, de Louw R, Oomen MHA, Arshad S, van den Bosch TPP, Dekker LJM, Doukas M, Luider TM, Riegman PHJ, van Kemenade FJ, Clahsen-van Groningen MC. Cryo-Gel embedding compound for renal biopsy biobanking. Sci Rep. 2019;9(1):15250.

Zajec M, Langerhorst P, VanDuijn MM, Gloerich J, Russcher H, van Gool AJ, Luider TM, Joosten I, de Rijke YB, Jacobs JFM. Mass Spectrometry for Identification, Monitoring, and Minimal Residual Disease Detection of M-Proteins. Clin Chem. 2020;66(3):421-33.

Zajec M, Jacobs JFM, de Kat Angelino CM, Dekker LJM, Stingl C, Luider TM, De Rijke YB, VanDuijn MM. Integrating Serum Protein Electrophoresis with Mass Spectrometry, A New Workflow for M-Protein Detection and Quantification. J Proteome Res. 2020;19(7):1326-33.

Zajec M, Frerichs KA, van Duijn MM, Nijhof IS, Stege CAM, Avet-Loiseau H, Luider TM, De Rijke YB, Jacobs JFM, van de Donk NWCJ. Cerebrospinal fluid penetrance of daratumumab in leptomeningeal multiple myeloma. Hemasphere. 2020;4(4):e413.

Zajec M, van Duin M, Luider TM, van der Velden VHJ, Russcher H, Stingl C, Sonneveld P, Broijl A, Jacobs JFM, De Rijke YB, VanDuijn MM. Minimal Residual Disease in Multiple Myeloma: Sensitive Quantification of Serum M-protein Using Mass Spectrometry. *Submitted.*

Zajec M, Noori S, VanDuijn MM, Russcher H, Tintu AN, Broijl A, Jacobs JFM, De Rijke YB, Luider TM. Blood-based mass spectrometry for longitudinal M-protein monitoring in multiple myeloma. *Submitted.*

Zajec M, Kros JM, Dekker-Nijholt DAT, Dekker LJM, Stingl C, van der Weiden M, van den Bosch TPP, Mustafa DAM, Luider TM. Identification of barrier-specific proteins in the human brain. *Submitted*.

PhD portfolio

Name PhD student:	Marina Zajec
Erasmus MC department:	Neurology and Clinical Chemistry
PhD period:	June 2016 – September 2020
Promotors:	Prof. Dr. Y.B. De Rijke
	Prof. Dr. P.A.E. Sillevis Smitt
Co-promotors:	Dr. T.M. Luider
	Dr. J.F.M. Jacobs

Conferences

Year	Topic	ECTS
2017	6th ESLHO Symposium on MRD and Immune-monitoring in Hemato-Oncology	0,3
2017	Validation of Biomarkers	0,3
2017	11th Mass Spectrometry in Biotechnology & Medicine Summer School	2,1
2017	21st MolMed Day	1,3
2017	5th Daniel den Hoed Day	1,3
2017	EuBIC Winter School on Proteomics Bioinformatics	1,2
2018	ASMS - American Society for Mass Spectrometry Annual Conference	2,1
2018	22nd MolMed Day	1,3
2018	MSACL - Annual Congress in Clinical Mass Spectrometry	1,5

Courses

Year	Topic	ECTS
2016	Erasmus MC - Introduction in GraphPad Prism Version 7	0,3
2017	Erasmus MC - Biomedical Research Techniques	1,5
2017	Erasmus MC - Scientific Integrity	0,3
2017	Erasmus MC - Basic Introduction Course on SPSS	1
2017	Erasmus MC - Microscopic Image Analysis: From Theory to Practice	0,8
2018	ASMS - Peptides and Proteins in Mass Spectrometry	0,6
2018	MSACL - Data Science	0,6

Lectures

Year	Topic	ECTS
2016-2020	JNI lectures	3
2016-2020	Clinical Chemistry Work Discussions	1,3
2016-2020	Proteomics Work Discussions	1,3

Teaching

Year	Topic	ECTS
2018	Supervising Bachelor Student Research - Julaisa Sierra	5
2019	Supervising Bachelor Student Research - Somayya Noori	5

Biography

Marina Zajec was born on the 18th of May 1992. She grew up Brezova, a small village in the north of Croatia. Raised by parents who were teachers and being a science enthusiast since secondary school, Marina has taken part in Summer School of Science in Croatia and XLAB International Science Camp in Germany. These summer science programs have inspired Marina to perform research, so after obtaining a master's degree in pharmacy at the University of Zagreb, she applied for an Erasmus+ grant and performed an internship studying the proteome of the blood-brain barrier with dr. T.M. Luider (Department of Neurology, Erasmus MC) and prof. dr. J.M. Kros (Department of Pathology, Erasmus MC).

Her wish to stay in the Netherlands and continue her education was granted and Marina started a PhD under supervision of prof. dr. Y.B. De Rijke (Department of Clinical Chemistry, Erasmus MC), prof. dr. P.A.E. Sillevis Smitt (Department of Neurology, Erasmus MC) and dr. T.M. Luider. In collaboration with dr. J.F.M. Jacobs, Radboudumc, Nijmegen, Marina has developed mass spectrometry assays to measure minimal residual disease in multiple myeloma. Marina's grit and passion have led to successfully finalizing her PhD period and the results of that research are presented in this dissertation.

During her PhD period Marina has met her husband Nick van Huizen and together they are raising their daughter Roza in Rotterdam.

Warning

Be careful
not to walk small
under the stars!

May your whole body
be filled with
the dim light of the stars!

To have no regrets
when with the last glances
you part with the stars!

In your final hour
instead of dust
pass whole to the stars!

Opomena

Čovječe pazi
da ne ideš malen
ispod zvijezda!

Pusti
da cijelog tebe prođe
blaga svjetlost zvijezda!

Da ni za čim ne žališ
kad se budeš zadnjim pogledima
rastajao od zvijezda!

Na svom koncu
mjesto u prah
prijeđi sav u zvijezde!

Antun Branko Šimić