A personalized mass spectrometry-based assay to monitor M-protein in multiple myeloma patients (EasyM)

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Statement of translational relevance

Minimal residual disease, a measure of depth of response to treatment, has become an important parameter in assessing the disease burden in multiple myeloma. MRD status has been correlated to PFS and OS in the clinic. Currently, the assays used to measure MRD require painful bone marrow aspiration, which prevents frequent sampling. In this study we demonstrated that residual levels of M-protein can be accurately and sensitively monitored in serial serum samples by mass spectrometry. The new assay, called EasyM, does not require bone marrow aspiration and thus can be performed frequently to monitor the disease status in CR patients and predict the relapse early. Comparison of EasyM to MFC with $10^{-4}$ sensitivity highlighted the superior performance of EasyM.
Abstract

Purpose: M-protein is a well-established biomarker used for multiple myeloma (MM) monitoring. Current improvements in MM treatment created the need to monitor minimal residual disease (MRD) with high sensitivity. Measuring residual levels of M-protein in serum by mass spectrometry (MS) was established as a sensitive assay for disease monitoring. In this study we evaluated the performance of EasyM – a non-invasive, sensitive, MS-based assay for M-protein monitoring.

Experimental Design: Twenty-six patients enrolled in MCRN-001 clinical trial of 2 high dose alkylating agents as conditioning followed by lenalidomide maintenance were selected for the study. All selected patients achieved CR during treatment, while 5 experienced progressive disease on study. The M-protein of each patient was first sequenced from the diagnostic serum using our de novo protein sequencing platform. The patient-specific M-protein peptides were then measured by targeted MS assay to monitor the response to treatment.

Results: The M-protein doubling over 6 months measured by EasyM could predict the relapse in four out of five relapsed patients 2-11 months earlier than conventional testing. In 21 disease-free patients, the M-protein was still detectable by EasyM despite normal FLC and MRD negativity. Importantly, out of 72 MRD negative samples with CR status, 62 were positive by EasyM. The best sensitivity achieved by EasyM, detecting 0.58 mg/L of M-protein, was 1000- and 200-fold higher compared to SPEP and IFE, respectively.
Conclusions: EasyM was demonstrated to be a non-invasive, sensitive assay with superior performance compared to other assays, making it ideal for MM monitoring and relapse prediction.
Introduction

Multiple Myeloma (MM) is a hematologic malignancy characterized by the proliferation of plasma cells in the bone marrow; it is often accompanied by CRAB features (hypercalcemia, renal insufficiency, anemia, and bone lesions) (1). In most MM patients, malignant plasma cells produce either intact immunoglobulin (Ig) or free light chain (FLC) (2). Thus, the secreted product of plasma cells, called M-protein or paraprotein, has long been used as a biomarker to monitor MM disease status, response to treatment, and relapse (3). At the time of diagnosis, the M-protein concentration in serum is typically higher than 30 g/L (2), but over the course of treatment the responders’ M-protein levels drop several orders of magnitude due to the reduction of clonal plasma cells in the bone marrow. Several diagnostic tests are currently employed in the clinic. Serum protein electrophoresis (SPEP) and immunofixation electrophoresis (IFE) quantify and characterize the M-protein based on the detection of the monoclonal protein on the gel or membrane with the respective detection limits of 0.5 g/L and 0.1 g/L (4). Serum FLC assay relies on nephelometry to measure the abundance of kappa and lambda FLC and their ratio with the sensitivity of less than 0.001 g/L (5). These methods are inexpensive, widely available, and appropriate for routine use. However, recent development of highly effective combination therapies has resulted in the high rates of complete responses (CRs) marked by the absence of detectable M-protein by IFE, and thus, requires the development of highly sensitive approaches to better estimate and monitor minimal residual disease (MRD) (6). Moreover, introduction of highly effective monoclonal Ab-based therapeutics poses additional challenges with CR
assessment as these can be detected as individual monoclonal bands in serum IFE (7,8).

MRD, a measure of the depth of response to therapy, has a prognostic value and has been correlated with progression free survival (PFS) and overall survival (OS) in the clinic (9). MRD assessment is currently performed with multiparameter flow cytometry (MFC) or next generation sequencing (NGS). These methods estimate the tumor burden by the quantification of cell-based parameters, such as the expression of aberrant cell surface markers (MFC) or Ig gene rearrangements (NGS), detecting as few as 1 tumor cell in 1,000,000 nucleated cells (9). However, these methods are costly and require painful bone marrow aspiration. In addition, bone marrow-based tests are subject to false negative readings due to the heterogeneous tumor cell infiltration and extramedullary disease (10).

Thus, there is a need for MRD assays that are reliable, non-invasive, cost-effective, and sensitive (6). Two mass spectrometry (MS)-based approaches have been proposed to meet this need by monitoring M-protein in the serum samples: an intact protein method and a clonotypic peptide method (11). Both approaches take advantage of the unique molecular mass of the M-protein. In the intact protein method, the mass of the M-protein’s heavy or light chains is determined in the diagnostic sample, where the M-protein amount is high. The specific intact mass is then monitored in the patient’s serum through treatment and relapse. The sensitivity of the intact protein method depends on the mass spectrometer used: LC-MS based monoclonal immunoglobulin rapid accurate mass measurements (miRAMM) method being more sensitive than MALDI-TOF-based Mass-FIX assay (12). Recently, the intact protein method was judged to be useful for
detection of relapse (13). However, the main limitation of this method remains the inability to detect M-protein below the concentrations of the polyclonal Igs (14). In the clonotypic peptide method, partial sequences of the M-protein are determined either though plasma cell mRNA sequencing (15,16) or through de novo peptide sequencing (17). The unique peptides are then used as a surrogate marker for the M-protein quantity in serum (15-18). The improved sensitivity of the clonotypic approach compared to the intact protein approach is due to the absence of interference from the polyclonal background (12).

The goal of this study was to evaluate the performance of EasyM – a non-invasive, sensitive MS-based personalized assay for M-protein monitoring. The assay represents a clonotypic peptide approach with full M-protein de novo sequencing and unique peptide quantification with parallel reaction monitoring (PRM). The study analyzed serial serum samples of 26 MM patients enrolled in the Myeloma Canada Research Network (MCRN)-001 study of augmented conditioning with busulfan and melphalan followed by lenalidomide maintenance. We report the sensitivity of EasyM and its ability to monitor treatment response and predict MM relapse.

Materials and Methods

MCRN-001 study design

The MCRN-001 study (ClinicalTrials.gov identifier: NCT01702831) is being conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice after approval by regulatory authorities and institutional review boards at each study site. Written informed consent was obtained for all study participants. A total of 78 newly diagnosed, transplant eligible MM patients
were induced with a bortezomib-based therapy before harvesting stem cells for autologous stem cell transplant (ASCT). Eligible MM patients received busulfan and melphalan conditioning prior to ASCT. Busulfan was administered IV at 3.2 mg/kg on days -5 to -3, or days -6 to -4 pre-ASCT (Day 0) and melphalan was given at 140 mg/m² on day -2 or -3 pre-ASCT. Lenalidomide maintenance therapy with 10 mg/d (increased to 15 mg/d after 3 months when appropriate) was initiated at 100 days (D100) post-ASCT and continued until progressive disease (PD).

The primary objective of the study was to determine the rate of MRD negativity by MFC ($10^{-4}$) at D100 post-ASCT. Secondary objectives included determination of the pattern of MRD analysis during lenalidomide maintenance, response rate using conventional Ig-based markers at D100 post-ASCT, and best response using lenalidomide maintenance. As an exploratory objective, 58 patients provided written consent for M-protein sequencing and monitoring with MS.

Serum and urine SPEP and IFE, and serum FLC were performed following standard clinical practice. Response assessments were completed according to the modified EBMT criteria and the recommendations for the uniform reporting of clinical trials (19).

For the purposes of primary and secondary endpoints, the MRD analysis by 8-color MFC ($10^{-4}$) was performed on serial bone marrow aspirates collected before induction therapy, before ASCT, on D100 post-ASCT, every 3 months for the 1st year, and then every 6 months until progression.

**Materials**
The following reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA):
dithiothreitol (DTT), iodoacetamide (IAA), trifluoroacetic acid (TFA), hydrochloric acid,
urea, acetone. HPLC-grade formic acid (FA) and acetonitrile (ACN) were from
ThermoFisher Scientific (Waltham, MA, USA). ProteaseMAX Surfactant, PNGaseF,
pepsin, trypsin, chymotrypsin, LysC, and AspN were from Promega (Madison, WI,
USA). Digestif standard protein was from Promise Proteomics (Grenoble, France).

**M-protein enrichment and enzymatic digestion for sequencing**

M-protein of IgG type was enriched from 10 μl of serum by Melon Gel IgG Purification
Kit (Pierce, Rockford, IL, USA), while M-protein of IgA type was enriched from 100 μl of
serum by Jacalin Sepharose (Abcam, Cambridge, UK). Both enrichments were
performed according to the manufacturers’ protocols. Jacalin-enriched sample was
desalted with Zeba™ Spin Desalting Columns (7K MWCO, ThermoFisher Scientific)
prior to further processing. A total of 100 μg of enriched sample was reduced in the
presence of 20 mM DTT for 15 min at 95°C and carboxymethylated with 30 mM IAA
for 30 min at room temperature in the dark. Reduced and alkylated proteins were
precipitated with cold acetone for 1 h at -20°C, then centrifuged for 10 min at 23000 g to
recover the pellet. The pellet was air-dried, resuspended in 4M urea, and incubated for
10 min at 37°C. The urea was diluted to less than 0.8 M with water and the samples
were equally divided into 5 parts. Each part (20 μg) was treated with one of the following
enzymes: pepsin (0.8 μg, for 45 min at 37°C, pH 2.0), trypsin, chymotrypsin, LysC,
AspN (1 μg of each enzyme for 6 h at 37°C). IgA-type M-proteins were treated with
PNGaseF (50 U/100 μg protein) for 90 min at 37°C prior to digestion. The pepsin
Digestions were stopped by boiling for 3 min at 95°C. All digests were dried in CentriVap concentrator (Labconco, Kansas City, MO, USA) and re-dissolved in 0.1% FA.

**M-protein digestion for LLoQ determination and quantification**

To establish the LOD and LLoQ for patient-specific peptides, the diagnostic serum sample (PI) was serially diluted into the control serum (H4522, Sigma-Aldrich) in 5-fold increments up to the final dilution of 78125-fold. The diluted serum was processed the same as the non-diluted time point series for each patient.

A volume of 0.83 μl of control or patient serum, diluted in 50 mM ammonium bicarbonate buffer, pH 8.0 and spiked with 0.5 μg of Digestif protein, was reduced with 25 mM DTT in the presence of 0.03% ProteaseMax surfactant for 15 min at 80°C with shaking and carbamidomethylated with 35 mM IAA for 15 min at room temperature in the dark. The excess of IAA was quenched by the addition of 3 μl of 250 mM DTT to 35 μl of reaction for 15 min at room temperature in the dark. The trypsin digestion was incubated in the presence of 0.03% ProteaseMax surfactant for 3 h at 37°C with shaking. The reaction was stopped by acidification with 10% TFA to a final concentration of 0.5% v/v. The digests were diluted to 0.02 μg/μl in 0.1% FA.

**Mass spectrometry**

A total of 0.5 to 1 μg of digested serum samples was loaded on EV-2001 C18 Evotips (Evosep, Odense C, Denmark) per manufacturer’s instructions. The peptides were separated on 15 cm (sequencing) or 8 cm (quantification) C18 column (PepSep, ReproSil 3 μm C18 beads, 100 μm ID, 8 or 15 cm) with the proprietary Evosep gradient of 0.1%FA/ACN for 44 min (sequencing, 30 samples per day method) or 21 min.
(quantification, 60 samples per day method) (20). The eluted peptides were injected in-line to the Orbitrap Fusion™ Trubrid™ or Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer (ThermoFisher Scientific) for sequencing or quantification, respectively. For the ionization, stainless steel emitters (ID 30 µm, OD 150 µm, PepSep, Marslev, Denmark) were maintained at 2 kV. DDA mode with the following parameters was used for sequencing: the MS spectra were collected with orbitrap resolution of 60000, scan range of 400-2000 m/z, AGC target of 4e5, and max IT of 100 ms. MS/MS scans were performed in centroid mode with orbitrap resolution of 15000, quadrupole isolation window of 2 m/z, AGC target of 1e5, and max IT of 50 ms with HCD and EThcD fragmentation. For quantification, the MS scan was followed by the targeted MS/MS with the inclusion list containing patient specific and Digestif protein peptides. Targeted MS/MS spectra were collected in centroid mode with the following parameters: orbitrap resolution of 17500, quadrupole isolation window of 2 m/z, AGC target of 3e6, max IT of 100 ms, 27% collision energy for HCD fragmentation. Retention time (RT) scheduling with a 6 min window around the expected RT was used for Digestif peptides.

Data analysis

Proprietary REmAb® software was used for M-protein sequence assembly (21). PRM assays were analyzed using Skyline 20 software (22). The peak area of a target peptide was normalized on the peak area of the Digestif peptide with the closest RT. The measurement for diagnostic sample was set to 100%. The percent residual M-protein was calculated by dividing the measurements in the follow-up sample by that of the diagnostic sample and multiplying by 100. The specificity of the assay was tested by
applying the PRM assay of one patient to control serum and to diagnostic samples of other MM patients.

For each patient, except 001-038, the calibration curve was built by serially diluting the diagnostic serum into control serum and plotting the normalized peak area versus expected concentration of M-protein. The expected concentration of M-protein was calculated by dividing the SPEP measurements (in g/L) in the diagnostic sample (PI) by the dilution factor. The calibration curve was analyzed by the linear regression in Excel; the slope and the intercept were used to calculate the concentration. The LOD was defined as the lowest M-protein concentration where the ion distribution pattern was similar to that of the diagnostic sample and the mass error for individual transitions was less than 10 ppm. The LLoQ was defined as the lowest M-protein concentration where calculated values were within 80–120% of the expected values and the coefficient of variation (CV) of duplicate injections was less than 20%.

Results

**MM patient selection and M-protein sequencing**

MRD negativity has become an important goal of initial treatment of MM patients. The MCRN-001 study, a Phase 2 trial of 2 high dose alkylating agents as conditioning followed by lenalidomide maintenance, aimed to increase MRD negativity rate after ASCT. The trial was designed to evaluate serial bone marrow samples for MRD by 8-color MFC \(10^4\) obtained before and after ASCT and during maintenance therapy. The study was later amended to evaluate M-protein sequencing and monitoring with MS. Fifty-eight out of 78 patients provided written consent for the analysis of serial serum samples with MS. The M-protein sequences were assembled with our *de novo*
sequencing platform for 54 out of 58 patients (93%). For the current study, 26 patients were selected based on the following criteria: achievement of CR by conventional criteria of serum and urine IFE negativity, availability of diagnostic sample and at least 8 consecutive serum samples. LC-only MM patients were excluded from the analysis due to difficulties in deriving the M-protein sequence. At the time of our analysis, 5 out of selected 26 patients had experienced disease progression on study.

The diagnostic (pre-induction, PI) sample for each patient was enriched for M-protein and sequenced with REmAb®, our proprietary monoclonal antibody sequencing platform. For each patient, the enrichment strategy was based on the patient’s initial clinical assessment of Ig isotype by IFE (Table 1). Upon the enrichment, the M-protein was digested with 5 enzymes and subjected to the LC-MS/MS analysis. The full M-protein sequence was assembled based on the de novo sequencing of the overlapping peptides produced by the multiple proteolytic enzymes (21). Figure S1 shows the entire variable region of heavy chain (HC) and light chain (LC) of M-protein of patient 001-001. Table 1 shows all 26 heavy chain complementarity-determining region 3 (HCDR3) sequences of the patients’ M-proteins. Only HCDR3 region was selected to be displayed for each patient since this region usually contains the highest number of mutations. The sequence was obtained for the entire M-protein even when its concentration was as low as 2 g/L. The M-protein sequence was then matched to the closest HC and LC germline sequences. The amino acids that deviated from the closest germline were considered patient-specific mutations and the tryptic peptides containing these mutations were selected. The unique peptide list for each patient was evaluated to select only the quantotypic peptides, based on the accepted criteria (23): peptides
with missed cleavages and modifications were eliminated. The M-protein sequence provided on average 6 unique peptides per patient.

The determination of sensitivity of the quantification assay

The M-protein LLoQ and LOD were determined for 2-3 best quantotypic peptides for each patient. The PI sample was serially diluted in the control serum, digested with trypsin, and analysed with PRM assay on a Q Exactive™ Hybrid Quadrupole-Orbitrap™. The LLoQ was determined as the highest dilution at which the observed amount deviated from the expected amount by less than 20% and CV of duplicate injections was less than 20%. Figure 1 shows the typical peak shapes of the unique fragments of peptide AEDTAFYYCAK detected at the diagnostic sample of patient 004-069 (A), where the M-protein was 30.4 g/L, as well as at the level of LLoQ, where the M-protein was 1.95 mg/L (B), but not in the diagnostic sample of patient 001-001 (D). Table 1 lists the lowest LOD and LLoQ values for all patients. The LLoQ differed dramatically in a peptide-specific manner with the highest sensitivity of 0.58 mg/L observed for peptide ADDTAVYYCVR. Although several unique peptides were identified for each patient, only the peptide with the lowest LLoQ was used for M-protein monitoring.

M-protein monitoring and relapse detection

To monitor the M-protein from diagnosis through treatment and relapse, patient serum was digested with trypsin and analysed with the patient-specific PRM assay. The patient-specific peptides were normalized on the spiked-in protein standard peptides. The normalized value in the follow-up sample was then divided by the normalized value...
of M-protein in the diagnostic sample to produce percent residual M-protein value. The
typical percent residual M-protein monitoring curve for disease-free (CR) patients is
shown in Figure 2. The continual drop of the M-protein amount measured with EasyM is
consistent with the M-protein values reported by the SPEP and IFE (compare the graph
to the clinical data listed in the table below). The high sensitivity of EasyM allowed the
M-protein to be detected and quantified even when the quantification was not possible
with conventional assays (Figures 1C, 2).

Most importantly, the improved M-protein detection by EasyM compared to SPEP and
IFE, allowed for early and accurate detection of relapse (Figure 3). The serial samples
of four patients with the confirmed progressive disease status were analyzed with
personalized PRM assays. The characteristic curve reflecting the amount of M-protein
was built for each patient as described above. A minimum of 2-fold increase in M-
protein level in any 2 consecutive tests 6 months apart was considered an indication of
a relapse by EasyM. Out of the five relapsed patients, the relapse was detected by
EasyM on M24 post-ASCT for patient 001-028, M18 for 004-045, M42 for 004-064 and
M19 for 006-023 (Figure 3), when the conventional analysis indicated that the patients
were still in CR (see the clinical data below each graph). Thus, by EasyM the relapse
was detected 5, 10, 11, and 2 months earlier than by the conventional analysis for
patients 001-028, 004-045, 004-064, and 006-023, respectively. Interestingly, the
change in the MRD status from Negative to Positive has coincided with or preceded the
M-protein increase detected by EasyM for all four patients. The fifth patient (002-003)
relapsed on M11 post-ASCT but had M-protein doubling detected by EasyM only on
M24 (Supplementary Table 1). It is worth noting that this patient had an M-protein
concentration of 6.3 g/L at the time of diagnosis (Table 1). At the time of relapse, a faint biclonal band was detected by the SPEP/IFE. Thus, it is speculated that the patient had a clonal switch, which explains our inability to predict the relapse.

To study the specificity of relapse detection, all time points with CR status and at least 12 months prior to the latest checkup were examined for progression-free patients. Out of the 21 patients, there were 121 time points satisfying the selection criteria. EasyM detected an at least 2-fold increase for only 2 of the 121 time points, corresponding to a 98.3% specificity. The increase in M-protein occurred from M12 to M18 for patient 004-061 and from M36 to M42 for patient 004-063 (Supplementary Table 1). For patient 004-061 the increase in M-protein was transient and maintained for another 6 months before decreasing by M30. For patient 004-063 the increase in M-protein amount was persistent until the last follow-up time point available (M60). We suspect that for patient 004-063 this increase in M-protein was due to permanent increase in the malignant plasma cell pool rather than interference from the polyclonal background, since two M-protein-specific peptides demonstrated the same trend. The transient increase in M-protein in patient 004-061 is difficult to explain since only one unique peptide was monitored, but, interestingly, similar transient increase was observed for kappa FLC measured by serum FLC assay.

For another 3 out of the 21 progression-free patients, EasyM could detect at least a 2-fold increase in M-protein level from the samples that were less than 12 months before their latest checkup. However, the future outcome of these patients is unknown at the time of our analysis. Therefore, it is hard to determine whether these are false positives or an indication of the upcoming relapse of these patients.
Overall, EasyM could detect M-protein in 88.89% of the available time points with confirmed CR status (Table 2). In contrast, among the available CR time points only 63.25% had abnormal FLC values and 46.27% were positive by MFC $10^{-4}$. Importantly, out of 62 MRD positive patient samples with CR status, only 6 samples were negative by EasyM, while out of 72 MRD negative samples, 62 were positive by EasyM (Supplementary Table 1). This result suggests that EasyM could detect the residual disease with greater sensitivity than MFC $10^{-4}$.

Finally, we compared the PFS of IFE-negative CR patients with (n=21) or without (n=5) detectable M-protein by EasyM. At a median follow-up of 69.5 months all five patients without detectable M-protein remained progression free whereas 28.6% (6/21) of patients in CR but with a measurable M-protein had progressed (Figure S2). Although the data are promising for the use of EasyM as a non-invasive assay for predicting outcomes, the difference between these two groups did not meet statistical significance (p=0.1720), likely as a result of the smaller sample size and paucity of progression events at the time of the analysis.

Discussion

In this study we evaluated EasyM, the personalized blood-based test to monitor M-protein with high specificity and sensitivity. The test consists of two steps (Figure 4): in the first step, the diagnostic serum sample is digested with multiple proteases to determine the full sequence of the M-protein. Unique tryptic peptides are then selected for each patient. In the second step, diagnostic as well as follow-up serum samples are digested with trypsin and analyzed with the PRM assay to determine the amount of M-protein at the subsequent time points relative to the diagnostic sample. The reported
value of percent residual M-protein can then be used to assess the disease status, monitor response to treatment, or predict disease relapse.

EasyM relies on the full amino acid sequence of M-protein obtained through *de novo* protein sequencing rather than plasma cell mRNA sequencing. The main advantage of such an approach is the ability to determine the M-protein sequence without painful, anxiety-provoking bone marrow sampling. Not infrequently, genetic material extracted from enriched samples cannot be used for sequencing due to suboptimal tumour content or low cellular yield. In addition, protein sequencing can be performed from diagnostic serum samples stored at -20°C for more than 7 years. To the contrary, the long-term storage of bone marrow-derived plasma cells would require liquid nitrogen to preserve the mRNA for sequencing.

EasyM can benefit MM patients if their M-protein at diagnosis is 2 g/L or higher and has at least one unique tryptic peptide for monitoring. At concentrations below 2 g/L, our current version of REmAb software can no longer distinguish the peptides obtained from the M-protein and the polyclonal background. Thus, the M-protein sequence cannot be assembled. This concentration requirement is not needed for the follow-up samples.

EasyM is suitable for monitoring M-proteins of IgG and IgA isotypes (Figures 2B-D and 2A, respectively). While IgG and some IgA-type M-protein can be monitored by SPEP, most IgA and some IgG type M-proteins migrate in the beta-region of SPEP resulting in inability to accurately quantify the M-spike due to interference from other abundant proteins in this region (24). For these patients, nephelometry-based quantification of total IgA is recommended (25). The broad applicability of our assay can more accurately quantify the clonal fraction of IgA in these patients.
EasyM is highly specific since unique patient-specific peptides are monitored. The recent introduction of effective mAb therapeutics complicates the disease monitoring in patients with M-protein characteristics similar to those of the drugs (8). To solve this problem, the HydraShift assay for daratumumab-treated patients has been developed (26). In addition, MS-based assays have proven their ability to distinguish between M-protein and mAb drugs (16,27-29). EasyM is almost completely proof to the interference from mAb therapeutics since such interference would require the entire variable region of the heavy and light chains to be identical to that of a drug, leaving no unique peptides for monitoring. This scenario is highly unlikely.

The main advantage of EasyM is its high sensitivity compared with other standard-of-care assays. We demonstrated up to 1000- and 200-fold higher sensitivity compared to SPEP and IFE, respectively. In addition, comparison of EasyM to FLC and MFC established superior performance (Table 2), although it must be noted that substandard MFC with $10^{-4}$ sensitivity was used in the study. High sensitivity combined with the non-invasive nature of our test make it ideal for continuous monitoring of MM patients in CR. Notably, EasyM was able to detect disease progression in 4 of the 5 patients 2-11 months earlier than conventional assays, such as SPEP and IFE. However, EasyM non-specifically detected doubling of M-protein over two consecutive time points in 2 out of 21 non-progressing patients. One of these doublings is a transient blip and the other is persistent until the last follow-up time point available. The false prediction of relapse can be improved by considering more frequent sampling over longer time interval. In addition, results of other sensitive complementary assays, such as NGS or MFC can help guide the treatment decisions in this case.
In recent years, MS-based assays play increasingly greater role in MM disease assessment. The IMWG Mass Spectrometry Committee recommended the detection of M-proteins by intact protein MALDI-TOF method instead of IFE and encouraged further studies on the use of MS-based assays for MRD assessment in blood (30). Intact protein assays demonstrated equal or superior performance when compared to the NGS assay with $10^{-5}$ sensitivity (31). It would be of great interest to compare EasyM performance to that of intact protein MS-assays. To date, the reported sensitivity for LC-MS-based miRAMM is lower than for EasyM (LLoQ is 0.05 g/L and 0.00058 g/L, respectively) (30). Thus, we speculate that EasyM will have similar, if not better, predictive value in MRD assessment from blood compared to intact protein assays. In addition, EasyM reports not only qualitative (detected/non-detected), but also quantitative M-protein values, providing more information on the disease status and trends than qualitative assays. However, it must be noted, that sample preparation time as well as cost is higher for EasyM due to the need for initial sequencing and enzymatic digestion. In addition, EasyM is more prone to false negative results due to clonal change than intact protein-based assays (11), although this limitation can be remediated by improvements in sequencing as discussed below.

Comparison of EasyM to other MS-based clonotypic peptide assays described in the literature revealed the similarities in the reported LLoQ and LOD: the best LOD of 0.026 mg/L achieved by EasyM for peptide GLEWMGVIYPGDSDTR (Table 1) is comparable to 0.5 mg/L reported for peptide GLEWVSYISSGGSTYYADSVK by Zajec et al (28), although LOD varied largely depending on the selected peptide in both assays. The main advantage of EasyM over the other clonotypic peptide-based assays is its label-
free format. EasyM does not require the synthesis of isotopically labeled unique peptides for M-protein quantification and instead uses a label-free protein standard to account for variations in digestion, sample loading, and MS performance. This allows EasyM to become a cheaper alternative to isotope-labeled assays with faster turnaround time.

As is the case with all M-protein monitoring tests, EasyM has its limitations. The non-secretory MM subtypes cannot be monitored due to the absence of detectable M-protein. LC-only patients, representing 20% of all MM cases (2), usually contain low amounts of M-protein, often not sufficient to derive the full sequence. This is a significant limitation, which will be addressed by the development of a LC-specific enrichment strategy. Our preliminary efforts on LC enrichment allowed sequencing of the M-protein in some, but not all LC-only patients (our unpublished data). Another limitation is a long half-life of Igs (23 days for IgG and 5.8 days for IgA) (4): their concentration in serum might not accurately reflect the real-time size of the plasma cell clone (32,33). Thus, the M-protein might be present even in the complete absence of the plasma cell clone secreting it (33). This must be considered when comparing the results of EasyM to bone marrow-based assays, such as NGS and MFC. The depth of response to treatment at a single time point might be underestimated by EasyM. As a result, clinical trials aimed at achieving MRD negativity by MS might reach this end point later than they would if MRD was detected by bone marrow-based assays. However, the increase in M-protein can only be explained by the growing population of the plasma cells that are actively producing the protein. In accordance with this reasoning, changes in the amount of M-protein in the serial serum samples, determined by MS-based
assay, correlated with PFS; patients whose M-protein amount increased or remained stable over time had worse prognosis compared to those whose M-protein levels decreased (13). EasyM determined that doubling of the M-protein over a 6-month period can predict relapse.

We envision to further develop EasyM in several ways. The sequencing ability will continue to improve, allowing us to sequence and monitor more than one M-protein, thus improving the disease monitoring in patients with biclonal disease and in the cases of a clonal change. The sensitivity will be improved through the incorporation of an M-protein enrichment step in the workflow. Our preliminary results indicate that albumin depletion can significantly lower LLoQ and LOD (data not shown). The data suggest that M-protein negativity by EasyM may be predictive of outcomes in patients achieving IFE-negative CR, however this will need to be validated in large prospective clinical trials. There also is a need to compare EasyM to the highly sensitive but invasive NGS assay, keeping in mind that this comparison might be biased by limitations of both bone marrow-based assays and our assay, as discussed above.

In conclusion, we developed and validated a non-invasive, sensitive, personalized MS-based assay that is ideal for frequent monitoring of MM patients in CR.
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Authors’ Contributions

ML, ZM, PT, LY, BM, and ST conceived and designed the study; GP, DR, ST provided MM patient serum samples and clinical data; ML, KG, XX collected and analyzed the data; ML, ZM, BM, EA interpreted the data; CY, QL, BM, EA wrote computer programs to assist the data analysis; ML, EA created figures; ML wrote the initial manuscript. The final manuscript was approved by all authors.
References


### Tables

#### Table 1. Summary of de novo M-protein sequencing. The complete sequences of the heavy and light chains were obtained with de novo sequencing. Only the HCDR3 is shown along with the peptide used for quantification with its LOD and LLoQ values.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Isotype, IFE</th>
<th>M-protein diagnosis measured by SPEG, g/L</th>
<th>Isotype, EasyM</th>
<th>HCDR3 sequence</th>
<th>Quantotypic peptide sequence and origin</th>
<th>LOD, g/L</th>
<th>LLoQ, mg/L</th>
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<tr>
<td>001-001</td>
<td>IgG KA</td>
<td>29.2</td>
<td>IgG1 KA</td>
<td>DWGYRVVPGYGLDING</td>
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<td>3.61E-04</td>
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<td>IgG KA</td>
<td>63.6</td>
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<td>DGYVVQDSLAVSLGER</td>
<td>ITGGYNYK, LC</td>
<td>1.15E-04</td>
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<td>001-028</td>
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<td>GAGGASTPLDY</td>
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<td>&lt;1.15E-04</td>
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<td>003-039</td>
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<td>IgG1 KA</td>
<td>QLGSGSAWEAPDN</td>
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<td>16.00</td>
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<td>004-030</td>
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<td>ELPAAF</td>
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<td>004-045</td>
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<td>NILFVEHNSLR, HC</td>
<td>4.68E-04</td>
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<td>ARMGYFYYFDY</td>
<td>AGSISTDLAWYQKPGQAPR, LC</td>
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<td>IgG KA</td>
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<td>IgG KA</td>
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<td>IgG LAM</td>
<td>22</td>
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<td>7.04</td>
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<td>IgG1 LAM</td>
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<td>2.11E-03</td>
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<td>28.16</td>
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<td>10.05</td>
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</tbody>
</table>

NQ denotes the non-quantifiable data. LOD and LLoQ were not determined for patient 001-038 due to the absence of M-protein measurement by SPEG at the diagnostic sample. Underlined cysteine residues are carbamidomethylated. HC, heavy chain of M-protein; LC, light chain of M-protein.
Table 2. Comparison of EasyM to MFC and FLC for all available time points with confirmed disease-free (CR) status.

<table>
<thead>
<tr>
<th></th>
<th>Available for analysis</th>
<th>Positive</th>
<th>Percent positive</th>
<th>Negative</th>
<th>Percent negative</th>
<th>Missing data</th>
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</thead>
<tbody>
<tr>
<td>EasyM</td>
<td>171</td>
<td>152</td>
<td><strong>88.89</strong></td>
<td>19</td>
<td><strong>11.11</strong></td>
<td>0</td>
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<tr>
<td>MRD by MFC</td>
<td>134</td>
<td>62</td>
<td><strong>46.27</strong></td>
<td>72</td>
<td><strong>53.73</strong></td>
<td>37</td>
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<tr>
<td>Serum FLC</td>
<td>166</td>
<td>105</td>
<td><strong>63.25</strong></td>
<td>61</td>
<td><strong>36.75</strong></td>
<td>5</td>
</tr>
</tbody>
</table>

MRD was determined by 8-color MFC with the sensitivity of $10^{-4}$.

Serum FLC was considered abnormal (positive) if any of the 3 parameters (free kappa, free lambda, or the kappa/lambda ratio) was outside the reference ranges (34).
Figure legends

Figure 1. The extracted ion chromatogram for the tryptic peptide AEDTAFYYCAK unique to patient 004-069’s heavy chain. The peptide was detected in diagnostic (PI) sample (A), in 15625 times diluted PI serum (B), at M24 post-ASCT, when patient was at CR (C), but not in PI serum sample of patient 001-001 (D). Retention times in minutes and mass accuracy in ppm of the most intense fragment ion are shown above the peak. The images were generated and analyzed with Skyline 20 software.

Figure 2. M-protein monitoring in disease-free (CR) patients. The graphs show the M-protein levels relative to the diagnostic (PI) sample measured by MS for representative patients from the MCRN-001 clinical trial: A 001-042, B 004-067, C 004-069, D 007-049. Peak areas of patient-specific unique tryptic peptides were normalized against peak areas of spiked-in standard protein peptides and further normalized on PI sample values to get the percent residual M-protein. M-protein levels could be monitored by MS at all time points. S (screening) denotes the time point after bortezomib induction, D100 and M6-M72 denote the 100 days or specific month post ASCT, respectively. The tables below each graph list the response to treatment, MRD status determined by the MFC (10^-4), M-protein amount monitored via serum protein electrophoresis (SPEP), and immunofixation electrophoresis (IFE). VGPR, very good partial response. P and N denote positive and negative readings, respectively. NQ denotes the non-quantifiable data. Not applicable (n/a) is used to denote missing data.

Figure 3. Early relapse detection in CR patients. The graphs show the M-protein levels relative to the diagnostic (PI) sample measured by MS for the relapsed (PD) patients from the MCRN-001 clinical trial: A 001-028, B 004-045, C 004-064, D 006-023. Peak areas of patient-specific unique tryptic peptides were normalized as described in Figure 2. M-protein levels could be monitored by MS from diagnosis through CR to PD onset. In all 4 PD patients, a 2 to 20-fold increase in M-protein in 2 consecutive tests 6 months apart was detected by MS 2 to 11 months earlier than clinical confirmation of PD by conventional testing. S (screening) denotes the time point after bortezomib induction, D100 and M6-M53 denote the 100 days or specific month post ASCT, respectively. The tables below each graph list the clinical data as described in Figure 2. P and N denote positive and negative readings, respectively. NQ denotes the non-quantifiable data. Not applicable (n/a) is used to denote missing data.

Figure 4. Overview of the EasyM assay. In the first step of the assay, 100 µl of diagnostic serum is collected to determine the full M-protein amino acid sequence using our established REmAb® sequencing platform. In the second step of the assay, unique patient-specific tryptic peptides from heavy and light chains are measured with parallel reaction monitoring (PRM) assay throughout all time points.
Figure 1
Figure 2
Figure 3
Step 1: Sequencing Patient’s M-protein

Collection of Diagnostic Sample

100 µL serum

M-protein is dominant

LC-MS/MS

Identification of Patient-Unique M-Protein Sequences

Step 2: Personalized Assay

Collection of Follow-up Sample

50 µL serum

M-protein below conventional detection limit

Tracking Patient-Specific M-protein Sequences after Treatment

Monitoring of M-protein as an Indicator of Tumor Burden

Figure 4
A personalized mass spectrometry-based assay to monitor M-protein in multiple myeloma patients (EasyM)

Mariya Liyasova, Zac McDonald, Paul Taylor, et al.

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