Monitoring Minimal Residual Disease in Patients with Multiple Myeloma by Targeted Tracking Serum M-Protein Using Mass Spectrometry (EasyM)

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ABSTRACT

Purpose: We investigated both the clinical utilities and the prognostic impacts of the clonotypic peptide mass spectrometry (MS)-EasyM, a blood-based minimal residual disease (MRD) monitoring protocol in multiple myeloma.

Experimental Design: A total of 447 sequential serum samples from 56 patients with multiple myeloma were analyzed using EasyM. Patient-specific M-protein peptides were sequenced from diagnostic samples; sequential samples were quantified by EasyM to monitor the M-protein. The performance of EasyM was compared with serum immunofixation electrophoresis (IFE), bone marrow multiparameter flow cytometry (MFC), and next-generation flow cytometry (NGF) detection. The optimal balance of EasyM sensitivity/specificity versus NGF (10^{-5} sensitivity) was determined and the prognostic impact of MS-MRD status was investigated.

Results: Of the 447 serum samples detected and measured by EasyM, 397, 126, and 92 had time-matching results for comparison with serum IFE, MFC-MRD, and NGF-MRD, respectively. Using a dotp >0.9 as the MS-MRD positive, sensitivity was 99.6% versus IFE and 100.0% versus MFC and NGF. Using an MS negative cutoff informed by ROC analysis (<1.86% of that at diagnosis), EasyM sensitivity remained high versus IFE (88.3%), MFC (85.1%), and NGF (93.2%), whereas specificity increased to 90.4%, 55.8%, and 93.2%, respectively. In the multivariate analysis, older diagnostic age was an independent predictor for progression-free survival [PFS; high risk (HR), 3.15; 1.26–7.86], the best MS-MRD status (MS-MRD negative) was independent predictor for both PFS (HR, 0.25; 0.12–0.52) and overall survival (HR, 0.16; 0.06–0.40).

Conclusions: EasyM is a highly sensitive and minimal invasive method of MRD monitoring in multiple myeloma; MS-MRD had significant predictive ability for survival outcomes.

Introduction

Multiple myeloma is characterized by the neoplastic generation plasma cells from bone marrow, which interfere with hematopoiesis and immunologic function, leading to hypercalcemia, renal insufficiency, anemia, and bone lesions (1, 2). Although the prognosis of patients with multiple myeloma has substantially improved over the past decades due to the advancements of novel agents and upfront autologous stem cell transplantation (ASCT), multiple myeloma remains incurable (3–5). Patients with multiple myeloma who achieve deep remission after treatment can still relapse due to recrudescence of tumor cells remaining in bone marrow, termed minimal residual disease (MRD) (6–8).

MRD is the most relevant prognostic factor for multiple myeloma and achieving undetectable MRD is associated with improved progression-free survival (PFS) and overall survival (OS; refs. 9–12) among patients whether they are newly diagnosed or have relapsed disease (9–11, 13–19). For this reason, patients often receive ongoing MRD monitoring, most commonly with multiparameter flow cytometry (MFC), next-generation flow cytometry (NGF), or next-generation sequencing (NGS). However, these methods require costly and painful bone marrow aspiration, limiting their utility for repeated sampling, and are subject to the risk of false-negative results due to non-representative sampling of focally-distributed plasma cells and hemodilution (20). Using serum protein electrophoresis (SPEP) and serum immunofixation electrophoresis (IFE) to measure patients' peripheral blood (PB) M-protein levels is a more practical and minimally invasive way of monitoring MRD, but these methods do not have sufficient sensitivity to evaluate patients who achieve remission deeper than complete response (CR). Therefore, the International Myeloma Working Group (IMWG) recommended that MRD assessments should be further evaluated on the basis of the treatments' existing efficacy evaluation (21). Given that longitudinal MRD monitoring could help inform dynamic risk stratification and adjustments

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Translational Relevance

Monitoring minimal residual disease (MRD) is one of the most important aspects of multiple myeloma clinical management, although it is challenging to conduct ongoing assessments because the commonly used MRD monitoring techniques currently require invasive bone marrow aspiration. EasyM, a clonotypic peptide mass spectrometry (MS) approach with extremely high detection sensitivity, enables targeted, quantitative tracking of M-protein in patients with multiple myeloma using peripheral serum, permitting both minimally invasive and longitudinal MRD monitoring in patients with multiple myeloma. In our comparison of the performance of EasyM with the serum immunofixation electrophoresis (IFE), multiparameter flow cytometry (MFC), and next-generation flow cytometry (NGF), we verified that the EasyM has comparable sensitivity to NGF (10^{-5}). This study also demonstrated the valuable application of EasyM in early prediction of disease progression and prognostic evaluation, underscoring its potential for extensive practical applications for minimally invasive MRD monitoring in patients with multiple myeloma.

to treatment strategies (22–26), a highly sensitive and blood-based MRD monitoring method is much-needed for patients with multiple myeloma.

Mass spectrometry (MS) is capable of detecting M-protein levels with high sensitivity in PB, and thus has the potential to monitor MRD with minimal invasiveness (27–37). MS methods such as the intact protein method and the clonotypic peptide method are now recommended by the IMWG (38). The clonotypic peptide method identifies patient-specific M-protein clonotypic peptide sequences to target and quantitatively track them, even after achieving deep remission (39–43). However, its application has been primarily explored in the context of clinical trials. For example, the MS-based EasyM assay was 1,000- and 200-fold more sensitive than SPEP and IFE, respectively, for the detection of M-protein in patient serum samples, which allowed earlier identification of relapse in a trial (35). EasyM had superior performance to MFC at 10^{-4} sensitivity but was not compared at 10^{-5} sensitivity, and the prognostic value of EasyM MS-MRD was not assessed. As an extension of this work, we evaluated the clinical utility of blood-based MS for longitudinal M-protein monitoring during the disease course of multiple myeloma in the real-world setting. To this end, we analyzed serial serum samples of 56 patients with multiple myeloma and compared the MS results with those determined using IFE, MFC, and NGF to establish the concordance between different disease-monitoring methods. In addition, we determined the optimal cutoff value of EasyM MS negativity to balance the sensitivity and specificity of MS versus NGF and explored the prognostic impact of MS-MRD status.

Materials and Methods

Study design and participants

This is a retrospective study (ClinicalTrials.gov ID: NCT05536700). It used a prospectively maintained institutional database of patients in China with multiple myeloma, the National Longitudinal Cohort of Hematological Diseases-Multiple Myeloma (NICHE-MM; ClinicalTrials.gov ID: NCT04645199). Additional data elements were obtained or confirmed by review of patient medical records and follow-up phone calls. Written informed consent was obtained from all patients for use of their medical record data and PB serum samples for research. The study was conducted according to the Declaration of Helsinki and approved by the institutional review board of the Institute of Hematology and Blood Diseases Hospital (IHBDH) on October 26, 2021.

Patients in the NICHE-MM cohort were eligible for inclusion in this study if they met the following criteria: (i) patients with newly-diagnosed multiple myeloma (NDMM) admitted at the lymphoma and myeloma treatment center of IHBDH from October 2013 to June 2019; (ii) received relatively consecutive treatment, defined as ≥4 cycles of induction treatment; (iii) with diagnosed M-protein type of IgG or IgA; (iv) had initial serum M-protein quantification of ≥5 g/L; and (v) availability of serum samples at baseline and ≥2 timepoints post-treatment. Of the 61 patients who met the inclusion criteria, 1 patient was excluded due to having biclonal M-protein (IgGκ and IgAκ) and 4 patients were excluded due to insufficient time intervals in their consecutive serum sample collection. This left 56 patients who fulfilled all criteria and were included in the study.

All included patients had started treatment with ≥4 cycles of bortezomib-based or lenalidomide-based induction threapy followed by either ASCT or three additional cycles of consolidation therapy. Patients were then treated with lenalidomide or bortezomib maintenance for ≥1 year unless intolerance or continual progression were observed. Routine monitoring of treatment response was performed every two courses during induction treatment, before and 3 months after ASCT or post-consolidation treatment, approximately every 3 months during maintenance, and when patients began exhibiting signs of recurrence or progression. The patient response assessments were conducted using the IWMG response criteria (44). The study participant demographics were considered to be representative for the general Chinese population (Supplementary Table S1).

MS for detection of monoclonal Ig

Materials

The following reagents were purchased from Sigma-Aldrich (Shanghai Titan Scientific Co., Ltd.): dithiothreitol, iodoacetamide, ammonium sulfate, urea, and acetone. MS-grade formic acid (FA) and acetonitrile were from Thermo Fisher Scientific. ProteaseMAX Surfactant, PNGaseF, pepsin, trypsin, chymotrypsin, LysC, GluC, and AspN were from Promega.

M-protein enrichment, sequencing, and quantification by MS

A simple and universal method was used for M-protein enrichment, salting out by ammonium sulfate solution with gradient concentration. This method is low-cost and suitable for different M-protein types including IgG, IgA, IgM, and IgD. In brief, 10 μL serum was diluted with 10 μL PBS, and then saturated ammonium sulfate solution was added to 35% (mass percentage). Immunoglobulins including M-protein were precipitated. According to the SDS-PAGE results of the precipitated proteins, the approximate purity and M-protein type were obtained. The following M-protein digestion using different enzymes and mass analysis were consistent with previous reports (35). Quantification of M-protein by using MS was performed as previously described and detailed in the Supplementary Materials and Methods.

Data analysis

The M-proteins were *de novo* sequenced following the same procedure as described (35). Parallel reaction monitoring (PRM) assays

were analyzed using Skyline software. The peak area of a target peptide was normalized to that of the spiked-in SILuMAb's peptide with the closest RT. The measurement for diagnostic samples was set to 100%. The percent residual M-protein was calculated by dividing the level in the next consecutive sample by that of the diagnostic sample and multiplying by 100. Assay specificity was evaluated by applying the PRM assay of one patient to control serum and to diagnostic samples of other patients with multiple myeloma.

The LOD was defined as the lowest M-protein concentration where the ion distribution pattern was similar to that of the diagnostic sample. In other words, a dotp score of ≥0.9 was used as the threshold for limit of detection (LOD). The lower limit of quantification (LLoQ) was defined as the lowest M-protein concentration, where calculated values were within 80% to 120% of the expected values and the coefficient of variation (CV) of duplicate injections was less than 20%.

Identification of clonotypic peptide of personalized M-protein

A novel LC/MS-based proteomics method was used to detect PB-based MRD in patients with multiple myeloma before and after treatment, as illustrated in **Fig. 1**. In the M-protein sequencing step, salting out by ammonium sulfate solution with a gradient concentration was an efficient and universal method for all different M-protein types except for free light chain. Thus, SDS-PAGE was performed as a preliminary quality control for M-protein enrichment evaluation. In the M-protein quantification step, the SILuMAb was added to patient serum at a known concentration as an internal quantification standard. Compared with the Digestif previously used in (35), SILuMAb has similar physicochemical properties to the M-protein, and the similar solubility and enzyme digestion efficiency may potentially help improve quantitative accuracy and stability. Tryptic peptides that both overlap with one of the CDR regions and contain at least one mutation compared with the germline gene were regarded as clonotypic and evaluated for their quantification sensitivity. The M-protein LLoQ and LOD were determined for two to three of the best quantotypic peptides for each patient. The PI sample was serially diluted in the control serum, digested with trypsin, and analyzed 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 <20% and CV of duplicate injections was <20%.

Risk stratification by FISH

Purified $CD138^+$ plasma cells followed by interphase FISH (iFISH) and panels were performed as reported previously (45). High-risk (HR) patients were defined at diagnosis by any presence of cytogenetic abnormalities (CA) including t(4;14), t(14;16), and Del(17P) and 1q21gains/amplifications (1q21+; ref. 46); patients without any of these CAs were considered as standard risk (SR). The cut-off level for translocation, deletions, and gains was set at 10%. Besides, the International Staging System (ISS) and Revised International Staging System (R-ISS) were also employed for risk stratification at baseline.

Flow-based MRD monitoring

The longitudinal MRD assessments performed in this study were assessed by using two combinations of eight-color panel MFC during January 2013 to December 2017, with a sensitivity for MFC MRD negativity of at least 1×10^{-4} as reported previously (45). Beginning in January 2018, we employed NGF methods (Euroflow standard) and patients were considered to have undetectable NGF-MRD when phenotypically aberrant clonal PCs <20 after recording ≥2,000,000 events in a corresponding sample (a sensitivity for MRD negativity of at least 1×10^{-5}), the panels of NGF were detailed in Supplementary Table S2.

Statistical design and analysis

PFS was measured from the start of treatment to disease progression, death, or the last follow-up, whichever occurred first. OS was defined as the time period from the start of treatment to any-cause death or the last follow-up. Both PFS and OS estimates were calculated using the Kaplan–Meier method, and log-rank tests were used to

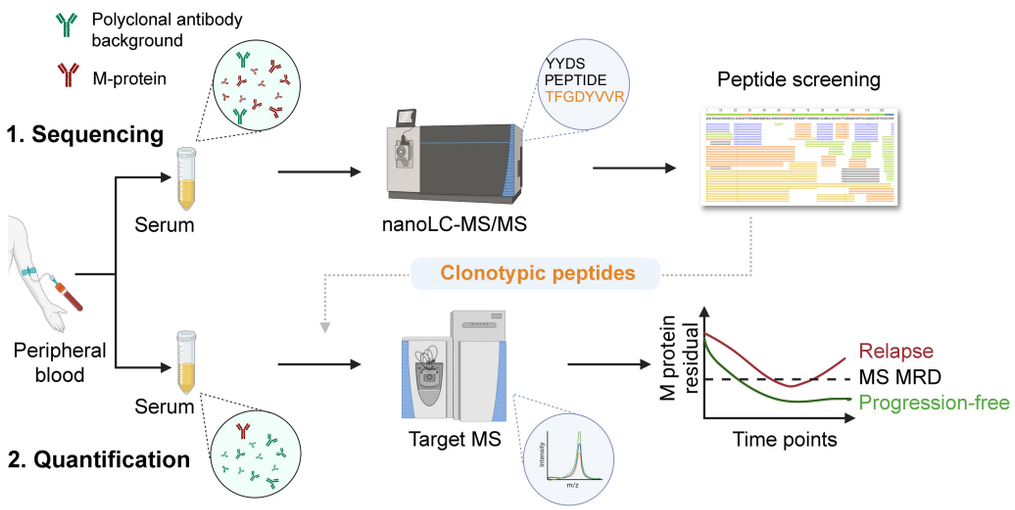


Figure 1.

The process of MS detection and quantification of M-protein. In Step 1, the M-protein was *de novo* sequenced from diagnostic serum of patients, then specific clonotypic peptides were selected for quantification. In Step 2, unique patient-specific tryptic peptides from heavy and light chains are measured with PRM assay throughout all time points. Created with BioRender (www.biorender.com).

compare the survival curves. ROC analyses were performed to set the best MS negative cut-off value to achieve the optimal balance of EasyM sensitivity and specificity.

HR was calculated using a univariate Cox proportional hazard model. Variables with $P < 0.1$ in the univariate Cox analysis were included in the multivariate Cox analysis. The prognostic impacts of MS-MRD status were evaluated in a multivariable Cox regression model including established risk factors. A $P < 0.05$ was considered statistically significant for the multivariable analyses. Statistical analyses were performed using SPSS (v25.0; IBM) and R (v4.1.2, Foundation for Statistical Computing) software.

Data availability

The raw data contain sensitive personal information such as patient-specific M-protein sequence, which are not authorized by the informed consent to share. However, the derived data that support the findings of this study are available from the corresponding authors upon reasonable request. To obtain access rights, the data requestor needs to sign the data access agreement.

Results

Characteristics, survival, and M-protein sequences of the study population

The patient baseline characteristics are presented in Supplementary Table S3. A total of 56 patients with NDMM [$n = 22$ (39.3%) female] were included in the analyses; the median age at diagnosis was 56 years. Thirty-eight patients had IgG type multiple myeloma and 18 had IgA type multiple myeloma. Forty-seven patients (83.9%) received bortezomib-based induction therapy and 44 (78.6%) received first-line ASCT. Half (50.0%) of patients had baseline risk stratification of ISS level II whereas 71.4% were RISS level II. The most common cytogenetic abnormalities were 1q21+, pos (51.9%); del (13q), pos (48.1%); and t (4;14), pos (30.4%). Among the entire population, the median follow-up time was 61.3 months, median PFS (mPFS) was 45.1 [95% confidence interval (CI), 31.0–59.1] months, and median OS (mOS) was not reached (NR; Supplementary Fig. S1).

Table 1 lists the patient-specific information for the M-protein of all 56 patients, including the baseline M-protein concentration measured by SPEP, the isotype, heavy chain complementarity determining region 3 (HCDR3) sequences, as well as the clonotypic peptides selected for quantification and their corresponding LOD and LLoQ.

Identification of personalized M-protein clonotypic peptide and longitudinal targeted monitoring

The identified clonotypic peptides overlapped with complementarity-determining regions. Supplementary Figure S2 shows a typical peak shape of the unique fragments of peptide NFVLLNWGR detected in a diagnostic sample from patient 23 (M-protein: 17.95 g/L; LLoQ: 1.15 mg/L), but not in the negative control serum sample. The lowest LOD and LLoQ values for all patients are listed in **Table 1**. The LLoQ differed substantially in a peptide-specific manner, with the highest sensitivity of 0.77 mg/L observed for peptide ASQSINLYVNWYQQRPGK. Although several unique peptides were identified for each patient, only the peptide with the lowest LLoQ was used for M-protein monitoring.

For the longitudinal monitoring of M-protein across the disease course, a patient's serum was digested with trypsin and analyzed with the patient-specific PRM assay. The patient-specific peptides were normalized on the spiked-in protein standard peptides, and the normalized value in the follow-up sample was divided by the normalized value of M-protein in the diagnostic sample to yield the percent residual M-protein value. The dynamic curve of M-protein residual (EasyM) for the 56 patients across the disease course and their corresponding clinical response are listed in Supplementary Table S4.

An example of the typical percent residual M-protein monitoring curve among disease-free (CR) patients is shown in **Fig. 2A**. The continual drop of the amount of M-protein measured with EasyM is consistent with the M-protein values detected by SPEP and IFE (see inset table in **Fig. 2A**). Furthermore, the high sensitivity of EasyM allowed for the detection and quantification of M-protein even when quantification was not possible with conventional assays. Of the 56 patients in the cohort, 25 were observed to have a similar curve dynamic with a steady decrease in M-protein over time (Supplementary Table S4).

Importantly, the improved sensitivity of M-protein detection provided by EasyM allowed for earlier and more accurate detection of relapse compared with SPEP and IFE. In this study, a minimum of a two-fold increase in M-protein level in any two consecutive tests, or a significant three-fold increase on a single test, was considered an indication of a relapse using EasyM. As shown in **Fig. 2B**, clinical response evaluation indicated that patient 29 (P29) was still in CR at timepoint 7 (T7), whereas a significant 3-fold increase in M-protein quantification was observed from T6 to T7. Not surprisingly, consistent with the prediction by EasyM, P29 experienced clinical progression 4 months post-T7. For patient 51 (P51; Supplementary Table S4), CR was maintained from T4 to T6, across 6 months. The quantitative value of M-protein detected by EasyM continually increased 3-fold from T4 to T6 for P51, whereas the bone marrow evaluation by NGF were negative at T6. However, P51 experienced disease progression at 12 months post-T6. This case further indicated that continuous dynamic trends may provide more accurate predictions than single timepoint detection. Early prediction of relapse by EasyM can also be observed for P19, P20, and P44 (Supplementary Table S4).

Patients within CR or poor response had high EasyM values at various time points, which were consistent with the conventional SPEP and IFE results (examples in **Fig. 2C** and **D**, respectively).

Methodologic comparison of MS-MRD with IFE, MFC-MRD, and NGF-MRD

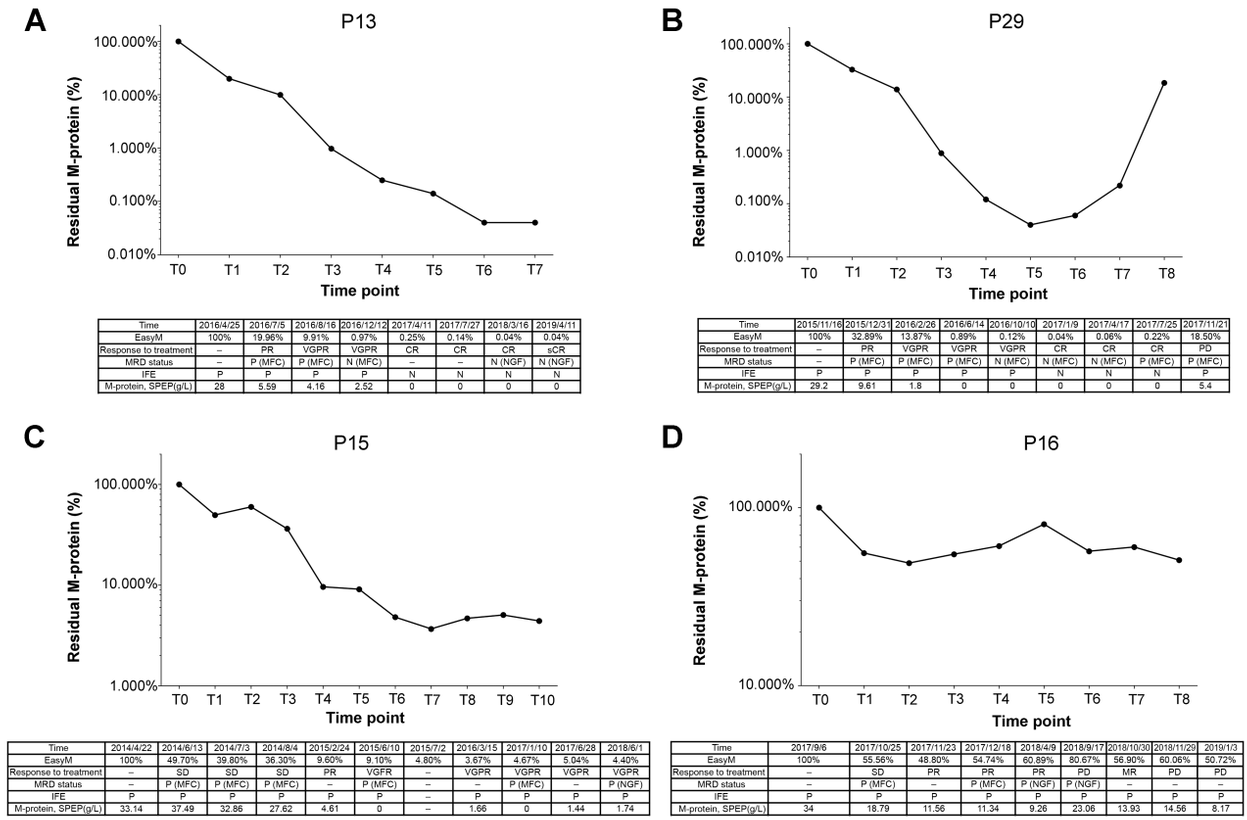
A total of 447 sequential serum samples from 56 patients with multiple myeloma were analyzed by EasyM. Of these samples, 397, 126, and 92 could be compared with IFE performed on serum samples, and MFC-MRD and NGF-MRD performed on bone marrow aspirates, at the same timepoints, respectively.

Using a dotp >0.9 as the MS-MRD positive, among the 397 comparable results between MS-MRD and IFE collected at the same timepoints, 70.8% (281/397) of the samples were both MS-MRD positive and IFE-positive, 3.3% (13/397) were both MS-MRD negative and IFE-negative, and 25.7% (102/397) were MS-positive and IFE-negative. Interestingly, there was one sample which was IFE-positive and MS-negative. To investigate this disagreement between methods, we further reviewed the patient's medical records and confirmed that the reason was due to the patient with IgAλ diagnostic M-protein and having an oligoclonal band of IgGκ after he achieved stringent CR. Overall, the sensitivity of MS-MRD was 99.6% in comparison with IFE, with 11.3% specificity (**Fig. 3A**), indicating that MS-MRD is more sensitive than a routine M-protein diagnostics protocol using IFE and provides enhanced ability to detect MRD-positive patients. Besides, we also performed analysis of sensitivity and specificity between

Table 1. Overview of *de novo* M-protein sequencing in the patient cohort.

Patient	M-protein by SPEP (g/L)	Type	HCDR3	Quantification peptide and location	LOD (mg/L)	LLoQ (mg/L)
P1	10.13	IgG1κ	ARLAVAGTRFYFDY	ASQDISNYLHWYQQKPGR, LCDR1	3.24	3.24
P2	39.12	IgG1λ	ASGLKCTGPSCPFDP	AGQAPVLVVYDDR, LCDR2	12.52	12.52
P3	14.02	IgG2κ	AKEEGDYGLDP	LVIYLGSNR, LCDR2	0.90	0.90
P4	11.36	IgG1κ	ARSEETKENEGFTVTAEG	ASQTILSYLNWYQQKPGK, LCDR1	3.64	3.64
P5	23.95	IgG1κ	AREVLSTSYYSYSYYMDV	QDGSETNYVDSVK, HCDR2	7.66	7.66
P6	9.20	IgAκ	MRPRELDGTNT	ELDGTNTWGQGTLVTVSSASPTSPK, HCDR3	2.94	2.94
P7	43.44	IgAλ	ARDRPPYKCGGGGYNGYCYNDV	VTISCSGSNSNIGGNAVTWYQR, LCDR1	0.56	2.78
P8	50.09	IgG1λ	ARGESSAAADRLGYYVMAV	QAPGQGLEWMGGIIPDVGVVK, HCDR2	3.21	3.21
P9	56.44	IgAλ	ARPREGYQLLRGGAAFDL	GLEWITIISNDGSQK, HCDR2	18.06	18.06
P10	36.29	IgG1λ	ARLRADCDRTTCLRGDAYLPDS	LLIYSHNQRPSGVPVR, LCDR2	2.32	2.32
P11	33.41	IgG1κ	ARDLYSGWYGLTG	LSCEASGFLFSSYAMHWVR, HCDR1	10.69	10.69
P12	34.28	IgAλ	VERQSSADGYSYFAN	RPSGIPGR, LCDR2	10.97	10.97
P13	28.00	IgG2λ	ARASSEGPLVPFDL	SVQWYQQKPGQAPVVVVHADSAR, LCDR2	8.96	8.96
P14	100.00	IgG1λ	AKDKTLAMAGTVYLES	SLSLSCAASGFTFDDYAMHWVR, HCDR1	32.00	32.00
P15	33.14	IgAκ	ARAASTYYHDSSVFGGSELDF	ASNLENGVPSR, LCDR2	10.60	10.60
P16	34.00	IgG2λ	ARGRYDSTSVFYGMDV	QAPGQGLEWMGWINTYTGK, HCDR2	10.88	10.88
P17	12.01	IgG1κ	ALRREPDYGDFHYFDP	ASQSINLYVNWYQQRPGK, LCDR1	0.15	0.77
P18	44.57	IgA2λ	ARAYSSGWPDHHFDY	GLEWIGSIFHSGSTYINPSLK, HCDR2	2.85	2.85
P19	41.16	IgAλ	THSPEAFRSEETKENEGFTVTAEG	LIIYDVSDRPSGVSNR, LCDR2	13.17	13.17
P20	44.25	IgAκ	VRRASMRQLYFYFYMDV	ASQTINTFLNWYQQKPGK, LCDR1	14.16	14.16
P21	37.47	IgG1κ	ARDSALSGLTYFDF	LLLYQASSLQPGAPSR, LCDR2	0.48	2.40
P22	21.76	IgAλ	ARLQGTPVAQMTEDAVNVERLT	VEAGDEAGYYCQVWDSSSDHR, LCDR3	6.96	6.96
P23	17.95	IgG1κ	ARDPRNFVLLN	NFVLLNWGR, HCDR3	0.23	1.15
P24	48.78	IgG1λ	ARALSYYGSGSYGWGWLDP	GLEWIGCINNSGSSNYK, HCDR2	15.61	15.61
P25	21.16	IgAκ	ARSEETKENEGFTVTAEG	VLIYAASSLQSGVPSR, LCDR2	6.77	6.77
P26	60.80	IgAλ	VRSPVGGVLGRTHFDY	LLIHDNDQRPSGLPDR, LCDR2	19.46	19.46
P27	47.37	IgG1κ	ARSLAVPTPQGGYYYGLDV	GLEWVLFISYDGSNEYYADSVK, HCDR2	15.16	15.16
P28	54.39	IgG1κ	ARQSLVQGVLRGFDY	GLEWVAVISYDGGNK, HCDR2	17.40	17.40
P29	29.20	IgG1λ	ARGNPGWFGEVNWFDP	GLEWIGTIYTVTYYNPSLK, HCDR2	9.34	9.34
P30	23.50	IgG2κ	VRLRDTTMLYPTDN	LLIYDVSNR, LCDR2	1.50	7.52
P31	10.45	IgG1κ	ARLRGSSSLEGDLSFDI	YSVNWYQQKPGEAPK, LCDR1	3.34	3.34
P32	33.36	IgG1κ	ARLGGSLTETTPPFDF	LEAEDVGLYYCMQGIDLPHTFGQGTK, LCDR3	10.68	10.68
P33	29.61	IgG1λ	AKVPVYELLTGAYGMDV	VEVGDEADYYCQVWDDSTDHWVFGGGTK, LCDR3	9.48	9.48
P34	57.23	IgG4λ	ARMKTTVTTPYRRPGNYGMDV	ALEWLAHIFSNDENSYSTSLR, HCDR2	18.31	18.31
P35	41.18	IgG3λ	ARGEKGCSGGRCFLDWFDT	QRPGQSPVLLIYQDNK, LCDR2	0.53	2.64
P36	19.14	IgAκ	VRGQWERSGFDLGFYLDY	WPSFGQGTK, LCDR3	1.22	6.12
P37	59.61	IgG1κ	VRGHDFLTGPFDY	ASQTVSSNLAWYQQKPGQAPR, LCDR1	0.76	3.82
P38	40.67	IgG1κ	AHLLATNTYYFDF	ASQFVGSWLAWYQQKPGTAPK, LCDR1	13.01	13.01
P39	54.59	IgG1κ	ARLGAGNSGDYPDY	LLIHDASTR, LCDR2	3.49	17.47
P40	16.28	IgAκ	ALDRQEWLGEKTCFDP	DSLNWYQQK, LCDR1	5.21	5.21
P41	52.94	IgG1λ	ARYVDWLWSAFDV	YDYVSWYR, LCDR1	16.94	16.94
P42	92.47	IgG1κ	ARETGRHSSLWHLDAF	LSCAASGFSFSTFGIHWVR, HCDR1	5.92	29.59
P43	40.29	IgG1λ	VREARGLLTFGGLLVPYFFDY	LSCAAAGFTFDNYNMNWVR, HCDR1	0.52	2.58
P44	40.60	IgG1κ	ARPPGGNFGTRPFDY	LSCAASGFTFNSYGMHWVR, HCDR1	12.99	12.99
P45	50.85	IgG1λ	ARDYMVATMRHGMDV	HGMDVWGQGATVTVSSASTK, HCDR3	3.25	16.27
P46	68.49	IgG1κ	ANKLGYCGSVSCHGWFDP	NSPFDFGPGTK, LCDR3	21.92	21.92
P47	66.80	IgG1λ	ARDGRRYDF	GLEWVSLIWSDATTK, HCDR2	4.28	21.38
P48	24.34	IgG1λ	ARHKGDSYDFDF	LGTEYACWYQQKPGQSPVLVIYEDSK, LCDR1	0.31	1.56
P49	49.79	IgG1κ	AKDLVAVAGTRRHCFDP	DLVAVAGTR, HCDR3	0.64	3.19
P50	56.91	IgG1λ	ARHSRGVATPFEY	DTERPSWIPDR, LCDR2	18.21	18.21
P51	47.46	IgG1κ	VRVFFDWLPSGGPFDP	ASQSVSDNLAWYQQKPGQAPR, LCDR1	0.61	3.04
P52	37.43	IgAλ	GSQRVMEWQALWLQY	GLEWIGSIHHSGK, HCDR2	2.40	11.98
P53	77.99	IgG1κ	SRARCSSTSCNFFHGMDV	SSGFSFGDYALTWVR, HCDR1	1.00	4.99
P54	60.52	IgAλ	SHYDWVFDS	AEDEADYYCSSYAGMNNFVVFGGGTK, LCDR3	19.37	19.37
P55	28.02	IgAκ	ARDRQNTALNPLLPAN	LSCTGSGFTFSSYGMHWVR, HCDR1	8.97	8.97
P56	15.34	IgG1κ	AKANWGGLDY	FLAWYQQKPGQAPSLLIYDASTR, LCDR1	0.98	4.91

Abbreviations: HCDR, heavy chain complementarity determining region; IgA, immunoglobulin A; IgG, immunoglobulin G; LCDR, light chain complementarity determining regions; LLoQ, lower limit of quantification; LOD, limit of detection; SPEP, serum M-protein electrophoresis.



Time	2016/4/25	2016/7/5	2016/8/16	2016/12/12	2017/4/11	2017/7/27	2018/3/16	2019/4/11
EasyM	100%	19.96%	9.91%	0.97%	0.25%	0.14%	0.04%	0.04%
Response to treatment	–	PR	VGPR	VGPR	CR	CR	CR	sCR
MRD status	–	P (MFC)	P (MFC)	N (MFC)	–	–	N (NGF)	N (NGF)
IFE	P	P	P	P	N	N	N	N
M-protein, SPEP(g/L)	28	5.59	4.16	2.52	0	0	0	0

Time	2015/11/16	2015/12/31	2016/2/26	2016/6/14	2016/10/10	2017/1/9	2017/4/17	2017/7/25	2017/11/21
EasyM	100%	32.89%	13.87%	0.89%	0.12%	0.04%	0.06%	0.22%	18.50%
Response to treatment	–	PR	VGPR	VGPR	VGPR	CR	CR	CR	PD
MRD status	–	P (MFC)	P (MFC)	P (MFC)	N (MFC)	N (MFC)	N (MFC)	P (MFC)	P (MFC)
IFE	P	P	P	P	P	N	N	N	P
M-protein, SPEP(g/L)	29.2	9.61	1.8	0	0	0	0	0	5.4

Time	2014/4/22	2014/6/13	2014/7/3	2014/8/4	2015/2/24	2015/6/10	2015/7/2	2016/3/15	2017/1/10	2017/6/28	2018/6/1
EasyM	100%	49.70%	39.80%	36.30%	9.60%	9.10%	4.80%	3.67%	4.67%	5.04%	4.40%
Response to treatment	–	SD	SD	SD	PR	VGFR	–	VGPR	VGPR	VGPR	VGPR
MRD status	–	P (MFC)	P (MFC)	P (MFC)	–	P (MFC)	–	–	P (MFC)	–	P (NGF)
IFE	P	P	P	P	P	P	–	P	P	P	P
M-protein, SPEP(g/L)	33.14	37.49	32.86	27.62	4.61	0	–	1.66	0	1.44	1.74

Time	2017/9/6	2017/10/25	2017/11/23	2017/12/18	2018/4/9	2018/9/17	2018/10/30	2018/11/29	2019/1/3
EasyM	100%	55.56%	48.80%	54.74%	60.89%	80.67%	56.90%	60.06%	50.72%
Response to treatment	–	SD	PR	PR	PR	PD	MR	PD	PD
MRD status	–	P (MFC)	–	P (MFC)	P (NGF)	P (NGF)	–	–	–
IFE	P	P	P	P	P	P	P	P	P
M-protein, SPEP(g/L)	34	18.79	11.56	11.34	9.26	23.06	13.93	14.56	8.17

Figure 2.

Representative patterns of disease remission defined by longitudinal M-protein monitoring by EasyM. **A,** Patient #13 with deep response; (**B**) patient #29 showed relapse after remission; (**C**) patient #15 with partial response, and (**D**) patient #16 with poor response. Abbreviations: MFC, multiparameter flow cytometry; PD, progressive disease; PR, partial response; sCR, stringent CR; SD, stable disease; T, time; VGPR, very good partial response.

EasyM and serum-free light chains (sFLC) ratio at the same timepoints ($n = 55$), the sensitivity of MS-MRD was 100% in comparison with sFLC ratio, with specificity of 2.5% (Supplementary Fig. S3A).

Flow cytometry MRD was performed on 218 bone marrow aspirates in this cohort, including 126 analyzed with MFC and 92 analyzed with NGF. MS-MRD detection was also performed on comparable serum acquired at the same time points. The sensitivity of MS-MRD was 100.0% in comparison with both MFC and NGF, with specificity of 1.9% and 22.2%, respectively (**Fig. 3B** and **C**), suggesting that MS-MRD also has higher sensitivity and ability to detect MRD real-positive patients compared with these testing modalities.

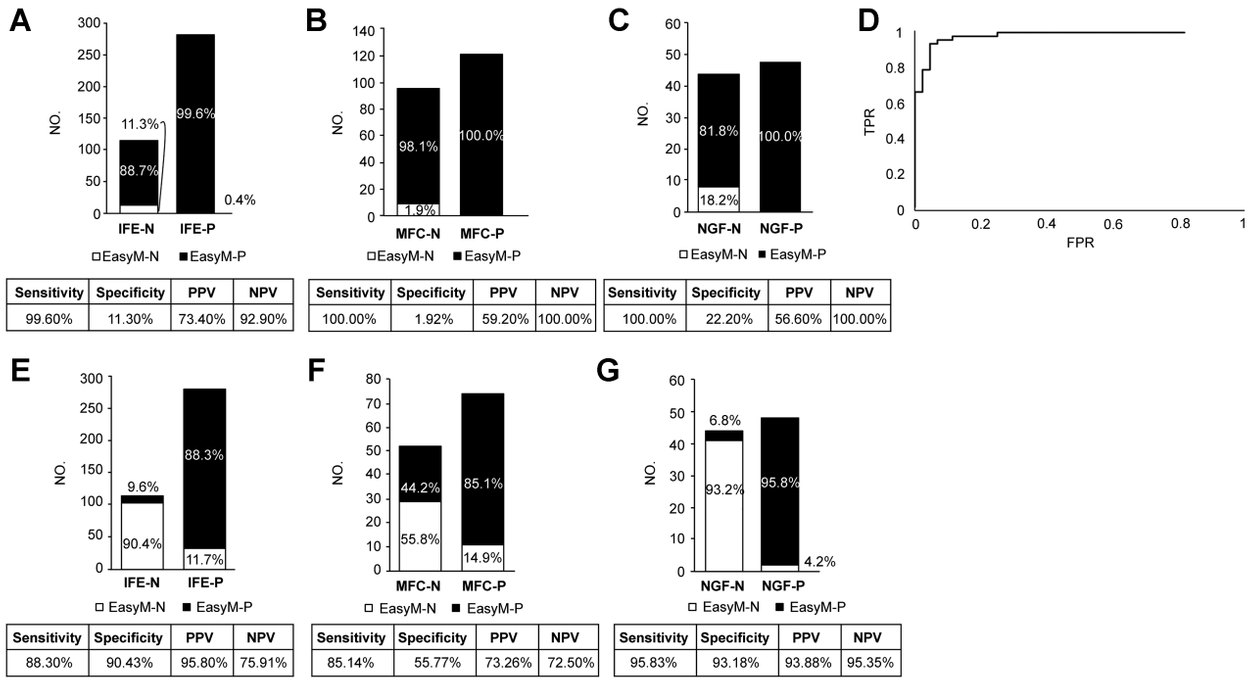
Optimal balance of EasyM sensitivity and specificity

Considering the overly high sensitivity of EasyM at a cutoff of dotp >0.9, we used the NGF as the standard for detection (i.e., a detection sensitivity $\leq 10^{-5}$) and set the EasyM MS negative cutoff value using ROC analysis, determined to be <1.86% of that at first diagnosis (**Fig. 3D**). Using these assumptions, we again performed the aforementioned analyses comparing the sensitivity and specificity between EasyM and IFE, MFC, and NGF. Under this scenario, the sensitivity of EasyM remained high in comparison with IFE (88.3%), MFC (85.1%), and NGF (93.2%), but the specificity was greatly increased to 90.4%, 55.8%, and 93.2%, respectively (**Fig. 3E–G**). Similar situations can also be found in the comparative analysis of easyM and sFLC ratio, the sensitivity of MS-MRD was 86.7% in comparison with sFLC ratio, with specificity dramatically increased to 75% (Supplementary Fig. S3b).

Prognostic impact of MS-MRD

First, all 56 patients were divided into MS-MRD positive ($n = 19$) and MS-MRD negative ($n = 37$) groups according to whether they achieved MS-MRD negativity, and mPFS and mOS were calculated for each group. The mPFS of the MS-MRD positive and MS-MRD negative groups was 20.6 (95% CI, 16.3–24.9) and 61.8 (95% CI, 41.6–82.0) months, respectively (**Fig. 4A**). The mOS was 55.7 (95% CI, 34.5–76.9) months for the MS-MRD positive group and not reached (NR) for the MS-MRD negative group (**Fig. 4B**). Both mPFS and mOS were significantly longer among the MS-MRD negative group compared with the MS-MRD positive group (both $P < 0.001$). The survival outcomes according to the best MS-MRD status were similar to those for the best NGF-MRD status (Supplementary Fig. S4).

Next, we investigated the prognostic impact of MS-MRD at two levels: prognostic utility of further risk stratification by combined best MS-MRD status and traditional response to treatment, and prognostic effectiveness of dynamic risk stratification by combined final MS-MRD status and baseline genetic risk stratifications. By combining best response with best MS-MRD status, five groups of patients were classified as sCR and MS negative ($n = 18$), CR and MS negative ($n = 15$), CR and MS positive ($n = 7$), ≤VGPR and MS negative ($n = 4$), and ≤VGPR and MS positive ($n = 12$). The mPFS of the groups were



Sensitivity	Specificity	PPV	NPV
99.60%	11.30%	73.40%	92.90%

Sensitivity	Specificity	PPV	NPV
100.00%	1.92%	59.20%	100.00%

Sensitivity	Specificity	PPV	NPV
100.00%	22.20%	56.60%	100.00%

Sensitivity	Specificity	PPV	NPV
88.30%	90.43%	95.80%	75.91%

Sensitivity	Specificity	PPV	NPV
85.14%	55.77%	73.26%	72.50%

Sensitivity	Specificity	PPV	NPV
95.83%	93.18%	93.88%	95.35%

Figure 3.

Performance of EasyM as compared with serum IFE and bone marrow MRD. Performance of EasyM as compared to (**A**) serum IFE, (**B**) bone marrow MFC, and (**C**) bone marrow NGF at EasyM MS negative cutoffs of dotp >0.9; (**D**) balanced cutoff value of EasyM sensitivity and specificity established by ROC analysis with NGF as the detection standard. Performance of EasyM as compared with (**E**) serum IFE, (**F**) bone marrow MFC, and (**G**) bone marrow NGF at EasyM MS negative cutoff <1.86% of that at first diagnosis. Abbreviations: FPR, false positive rate; N, negative; No, number; NPV, negative predictive value; P, positive; PPV, positive predictive value; TPR, true positive rate.

NR, 32.8 (95% CI, 22.8–42.8), 41.7 (18.8–64.6), 38.1 (0–77.2), and 17.0 (15.5–18.7) months, respectively ($P < 0.001$; **Fig. 4C**). The mOS were NR, NR, NR, 62.6 (95% CI, 0–130.6), and 43.5 (95% CI, 22.3–64.7) months, respectively ($P < 0.001$; **Fig. 4D**). Four groups of patients were identified according to their baseline genetic risk stratification and final MS-MRD status as SR and MS negative ($n = 8$), SR and MS positive ($n = 9$), HR and MS negative ($n = 26$), and HR and MS positive ($n = 11$). The mPFS was 49.9 (95% CI, 8.9–90.7), 18.9 (95% CI, 15.8–22.2), 60.8 (95% CI, 51.8–68.7), and 20.9 (95% CI, 26.8–57.2) months, respectively ($P = 0.0015$; **Fig. 4E**). The mOS was NR, 63.5 (95% CI, 28.4–98.5) months and NR, 55.6 (95% CI, 31.5–79.8) months, respectively ($P < 0.001$; **Fig. 4F**). HR and MS negative patients had significantly longer mPFS and mOS than those who were HR and MS positive (both $P = 0.002$). SR and MS negative patients had numerically longer mPFS ($P = 0.067$) and significantly longer mOS ($P = 0.038$) than those who were SR and MS positive.

Finally, to investigate whether EasyM MS-MRD status remained an independent predictor of PFS and OS when other prognostic factors (i.e., patients' diagnostic age, baseline ISS stage, LDH levels, risk stratification, and treatment options) were taken into account, we initially performed univariate analyses of PFS and OS. For PFS, the univariate analysis showed that both diagnostic age and best MS-MRD status were significant prognostic factors (both $P < 0.01$), and the multivariate analysis verified the independent impacts of older diagnostic age (HR, 3.15; 95% CI, 1.26–7.86) and best MS MRD status of MS-MRD negative (HR, 0.25; 95% CI, 0.12–0.52) on PFS (both $P < 0.05$; **Table 2**). For OS, diagnostic age, receiving upfront ASCT, and best MS-MRD status demonstrated stronger prognostic impacts in the univariate analysis (all $P < 0.1$). When including these established prognostic impactors into a multivariable analysis, the results revealed that only the best MS-MRD status of MS-MRD negative remained a significant independent factor for improved survival (HR, 0.16; 95% CI, 0.06–0.40; $P < 0.001$; **Table 2**).

Discussion

The two different strategies for M-protein detection by MS, include the intact protein method and clonotypic peptide method (41, 43, 47). Briefly, the intact method is rapid and high-throughput using MALDI-TOF MS, but it is more affected by the polyclonal background. Marion and colleagues compared the performance of MALDI-TOF MS head-to-head with an established MRD assay by flow cytometry, and the results suggested that MALDI-TOF MS adds value to bone marrow-based MRD testing (27). Puig and colleagues demonstrated that, in comparison with IFE, EXENT and FLC-MS are better able to identify and characterize the M-protein of patients with multiple myeloma in baseline samples and detect residual disease in a higher proportion of cases during treatment monitoring, to more accurately predict patients' outcome (30). Clonotypic peptide assay has ultra-high sensitivity although it is personalized and patient-specific, requiring advanced bioinformatic algorithms to obtain unique peptides for each patient. The methodology of EasyM is similar to that of the contemporaneous Sebia assay (48), using a spiked monoclonal stable isotope-labeled Ig as a reference, which provides an advantage in quantitative quality control.

This study is the first to evaluate the clinical and prognostic utilities of EasyM blood-based MS-MRD among a real-world cohort with multiple myeloma by comparing its sensitivity and specificity with

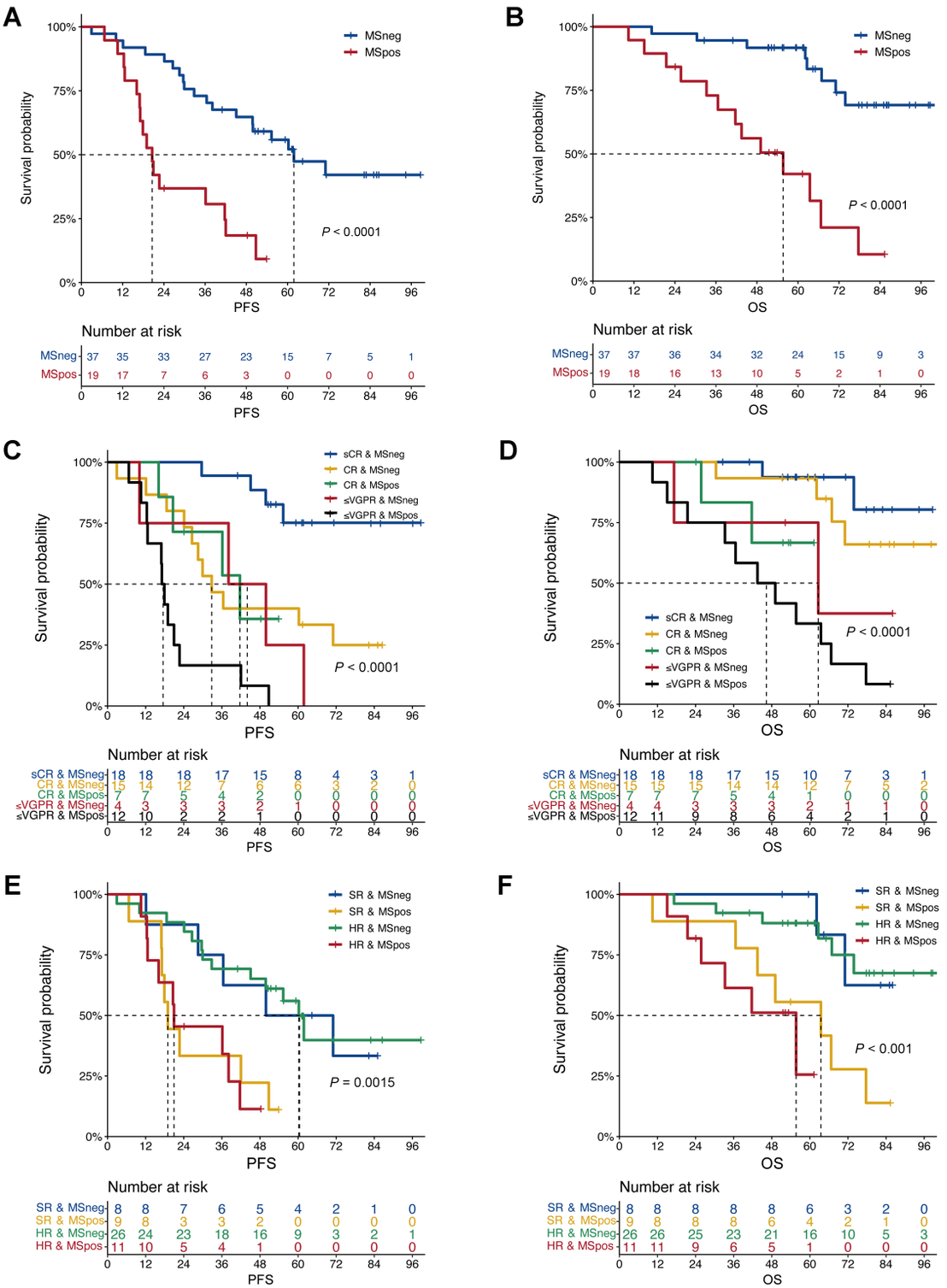


Figure 4.

Prognostic impact of EasyM MS-MRD (**A**) PFS and (**B**) OS by best MS-MRD status. **C,** PFS and (**D**) OS by best MS-MRD status in combination with best clinical response. **E,** PFS and (**F**) OS by final MS status in combination with baseline genetic risk stratifications. Abbreviations: HR, high risk; MRD, minimally residual disease; SR, standard risk.

Table 2. Univariate and multivariate analyses of prognostic impactors of PFS and OS.

	PFS				OS			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Diagnostic age								
≥65 vs. <65 years	3.39 (1.38–8.31)	0.008	3.15 (1.26–7.86)	0.014	2.36 (0.79–7.02)	0.045	1.89 (0.45–7.90)	0.383
ISS stage								
II vs. I	0.99 (0.33–2.98)	0.992	—	—	0.71 (0.20–2.60)	0.609	—	—
III vs. I	0.90 (0.29–2.78)	0.855	—	—	0.73 (0.19–2.75)	0.641	—	—
Elevated LDH								
Yes vs. No	1.15 (0.44–3.01)	0.773	—	—	2.61 (0.70–9.67)	0.153	—	—
Risk stratification								
HR vs. SR	1.36 (0.69–2.72)	0.376	—	—	0.69 (0.29–1.64)	0.403	—	—
Upfront ASCT								
Yes vs. No	0.60 (0.28–1.29)	0.191	—	—	0.43 (0.17–1.07)	0.070	0. 65 (0.19–2.18)	0.483
Best MS-MRD status								
MS neg vs. MS pos	0.24 (0.11–0.50)	<0.001	0.25 (0.12–0.52)	<0.001	0.16 (0.07–0.40)	<0.001	0.16 (0.06–0.40)	<0.001

Abbreviations: HR, high risk; ISS, International Staging System; LDH, lactose dehydrogenate; neg, negative; pos, positive; SR, standard risk.

conventional M-protein diagnostics and bone marrow MRD evaluation. The performance of EasyM over the clinical course of therapy was compared with that of IFE using consecutively collected peripheral serum samples and with MFC/NGF using time-matched bone marrow samples. Our findings indicated that EasyM had superior sensitivity for the detection of M-protein in comparison with serum IFE, particularly for the identification of patients in deep remission. Empirical adjustment to balance EasyM sensitivity with specificity, using an NGF detection sensitivity of 10^{-5}, led to the determination of an optimal cutoff of <1.86% of that at first diagnosis. Finally, the multivariate analysis revealed that only the best MS-MRD status was an independent predictor of both PFS and OS, underscoring the prognostic utility of EasyM for MRD monitoring.

Across comparisons with conventional M-protein detection methods, EasyM consistently demonstrated the advantage of ultra-sensitivity, which is of high importance for detecting low levels of M-protein secreted by residual plasma cells in multiple myeloma, where patients frequently relapse despite achieving CR. The response criteria in multiple myeloma have been recently amended and MRD is now considered the most crucial prognostic indicator of a subsequent outcomes (5, 25, 49). This underscores the urgent need for sensitive MRD evaluation methods capable of longitudinally monitoring tumor burden with higher frequency than bone marrow-based methods, whether directly or indirectly, to indicate disease trajectory and allow clinicians to identify disease recurrence earlier and make timely modifications to treatment regimens.

Over 68% of patients with multiple myeloma report bone pain and associated health-related quality of life decrements (50), which may lead to refusal of frequent bone marrow sampling. As a test requiring PB, EasyM provides the advantage of minimal invasiveness, which could promote patient compliance with the repeated testing required to form a comprehensive picture of their treatment response over time. Futhermore, EasyM takes about a comparable amount of time to identify M-protein in PB as NGF does to identify plasma cells in bone marrow (EasyM requiring 2 days for M-protein sequencing and 1 day for subsequent M-protein quantification, and NGF requires 1 day for detection and about 3 days for analysis and final report in our hospital). In addition, the MS cutoff values used in this study for EasyM were set at levels suitable for clinical applications, equivalent to a sensitivity of 10^{-5}, retaining the possibility of further comparisons with NGS-MRD. Indeed, there was some level of inconsistency between MS and the other detection methods in regard to true positives or true negatives. As EasyM has higher specificity compared with traditional IFE, EasyM monitoring can be especially helpful when oligoclonal interference occurs (35) or when patients receive monoclonal antibody therapy (51–54). In addition, when conventional M-protein tests and bone marrow MRD assessments yield inconsistent results (49, 55), the MS methodology could also provide valuable reference to help clinicians evaluate a therapy's efficacy and form clinical strategies accordingly.

Although it is currently difficult to assess treatment efficacy among patients with multiple myeloma with serum M-protein ≤10 g/L, the present results demonstrate that EasyM MS can target and quantitatively track M-protein even if the patient's initial levels are as low as 9.2 g/L. This is consistent with previous studies using MS to monitor M-protein in nonsecretory multiple myeloma (NSMM) and oligosecretory multiple myeloma (OSMM) patients, which suggest that MS may also play a key role in evaluating treatment efficacy among OSMM or NSMM patients in the future (30, 42). MS cannot currently replace bone marrow-based NGF-MRD entirely; however, an ideal approach for patients who are reluctant to undergo repeated bone punctures might be first performing an MS-based evaluation and then moving to bone marrow-based MRD evaluation after MS turns negative. This approach would reduce the pain of repeated bone punctures without sacrificing sensitivity of MRD detection, while maintaining the flow of clinical information to inform treatment strategies.

Because of delayed clearance of abnormal proteins in the blood, MRD is reached faster in bone marrow than as CR in PB (49, 56, 57). Future research with larger population sample size is warranted to further explore the optimal M-protein-based MRD-negative definitions. More accurate and reliable M-protein-based MRD cut-offs may be determined by taking the half-time of different kinds of immunoglobulin into account in addition to EasyM's measurement of M-protein, flow-based MRD detection, and evaluation of response to treatment. Novel statistical analysis methods like AI or deep learning algorithms may play a role in such comprehensive analysis. Although other blood-based detection methods including blood-NGF and cell-free DNA(cfDNA) are affected less by delayed clearance, they have their own drawbacks. For example, cfDNA cannot detect MRD when the fraction of circulating tumor DNA (ctDNA) in blood is below the

genomic equivalent limit (i.e., twice the inverse of the number of copies of each gene in a sample; refs. 47, 58). Blood-NGF has advantages in monitoring circulating tumor cells (CTC) but also had limitations in monitoring MRD as there is no clear correspondence between CTC in PB and MRD in bone marrow. Thus, neither EasyM nor blood-based NGF nor cfDNA can completely replace the assessment of bone marrow MRD currently, but each have their own advantages in assessing the tumor burden of multiple myeloma, which merit exploration and application in a reasonable clinical context.

The findings of this study should be interpreted with caution in the light of a few limitations. First, there is potential selection bias given the inclusion and exclusion criteria for this study. For example, only patients with M-protein IgG and IgA types were eligible for inclusion; thus, the results do not reflect the detection of IgD or light chain M-protein types. Second, the population of 56 patients is relatively small, and the available NGF and MS results with matching time points for comparison were also limited. Therefore, the presently obtained negative cutoff value of MS should be considered a preliminary conclusion and future studies with larger cohorts are recommended to further explore the optimal cutoff value. Future studies could include MS in prospective clinical trials for efficacy evaluations, assessment of the prognostic significance of MS at different time points through fixed interval time point monitoring, as well as the ability of MS to predict clinical relapse before it occurs. Third, because 98.2% (55/56) of patients had measurable lesions (serum MP >10g/L), we commonly performed serum/urine SPEP and IFE as efficacy evaluation tools. Thus, only 53.6% (30/56) of patients had sFLC detection to further confirm the status of sCR and disease progression, and many of the patients who had undergone sFLC testing also had missing sFLC test results at various time points. Thus, a future prospective study including longitudinal EasyM and sFLC monitoring in patients with multiple myeloma is also worth further exploration.

In conclusion, the results of this study demonstrate that MS is a minimally-invasive and highly sensitive M-protein detection method for blood-based MRD monitoring over the disease course of multiple myeloma, with substantial utility for the prognostic screening of patients who achieve deep remission following treatment. Using an empirically determined MS negativity cutoff, the optimal balance of sensitivity and specificity with EasyM could be achieved while maintaining superior sensitivity over conventional MRD detection methods.

Authors' Disclosures

No disclosures were reported.

Authors' Contributions

H. Fan: Conceptualization, data curation, formal analysis, investigation, visualization, methodology, writing–original draft, writing–review and editing. **B. Wang:** Conceptualization, resources, data curation, formal analysis, supervision, investigation, visualization, methodology, writing–original draft, writing–review and editing. **L. Shi:** Data curation. **N. Pan:** Data curation, software, formal analysis. **W. Yan:** Data curation. **J. Xu:** Data curation, software. **L. Gong:** Data curation, software. **L. Li:** Data curation, software. **Y. Liu:** Data curation, software. **C. Du:** Data curation, software. **J. Cui:** Data curation. **G. Zhu:** Data curation, software. **S. Deng:** Data curation, software, supervision. **W. Sui:** Data curation, software. **Y. Xu:** Data curation, software, supervision. **S. Yi:** Data curation, supervision. **M. Hao:** Software, supervision. **D. Zou:** Software, supervision. **X. Chen:** Supervision. **L. Qiu:** Conceptualization, resources, supervision, funding acquisition, project administration, writing–review and editing. **G. An:** Conceptualization, resources, supervision, funding acquisition, project administration, writing–review and editing.

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Note

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