Comparison of EasyM, a clonotypic mass spectrometry assay, and EuroFlow minimal residual disease assessment in multiple myeloma

Minimal residual disease (MRD) negativity is increasingly recognized as the optimal measure of therapeutic response in multiple myeloma (MM) and is associated with improved progression-free and overall survival.[1] Bone marrow (BM) MRD assessment with next-generation sequencing (NGS) or next-generation flow cytometry (NGF) can achieve a minimum sensitivity of 10^{-5},[2] however requires an invasive procedure. Additionally, this single-site BM biopsy may be limited by specimen quality and failure to capture MM's spatial heterogeneity.[3] Peripheral blood-based mass spectrometry (PB-based MS) can sequentially quantify unique amino acid sequences and unique molecular masses of monoclonal proteins (M-proteins),[4] offering the potential for highly sensitive, non-invasive sequential MRD assessment. Two MS methods, the clonotypic peptide and intact immunoglobulin light chain approaches, have been employed.[5] The clonotypic peptide approach has lower throughput but is personalized and highly sensitive, able to detect residual M-proteins at a level of 0.001 g/L.[6]

In this context, we retrospectively evaluated the PB clonotypic MS EasyM assay in MM patients undergoing sequential EuroFlow NGF MRD. MM patients enrolled in the Australasian Leukemia and Lymphoma Group MM19 (*ANZCTR.org.au identifier: ACTRN12616000772448*) and MM21 (*ANZCTR.org.au identifier: ACTRN12618001490268*) trials with measurable M-protein ≥2 g/L by serum protein electrophoresis and/or free light chains (FLC) ≥100 mg/L at baseline were identified. Briefly, the MM19 trial evaluated the addition of ixazomib (ITd) to thalidomide and dexamethasone (Td) consolidation for 12 months in transplant-eligible newly diagnosed MM patients undergoing front-line autologous stem cell transplantation (ASCT), whilst the MM21 trial evaluated an intensive salvage approach using daratumumab-lenalidomide-dexamethasone (DRd) as re-induction (DRd x 4) and post-ASCT consolidation (DRd x 12 followed by R maintenance until progression) in transplant-eligible MM patients failing (< partial response as best response) front-line bortezomib-based induction therapy.[7] EuroFlow NGF MRD status was determined from BM samples at pre-ASCT, post-ASCT, and end of consolidation time points in MM19 and MM21 and additionally post-cycle 2 of consolidation in MM21 (*Online Supplementary Figure S1*). Matched serum samples and additionally unmatched samples post-cycle 7 of consolidation in MM19 were evaluated with EasyM. Protocols were approved by ethics committees of all participating centers.

Utilizing the standardized 8-color EuroFlow platform,[8] samples were analyzed with a Navios flow cytometer (Beckman Coulter, USA) and Infinicyt software (Cytognos SL, Spain). Test sensitivity at a level of 10^{-5} was defined with thresholds for the limit of detection (LOD) of 20 cells in $2x10^6$ nucleated BM cells and the lower limit of quantification (LLOQ) of 50 cells in $5x10^6$ nucleated BM cells according to International Clinical Cytometry Society consensus guidelines.[9] Disease responses were assigned according to the International Myeloma Working Group consensus response criteria.[2]

EasyM, a commercial PB clonotypic MS assay (Rapid Novor, Canada), involves *de novo* amino acid sequencing of the full-length M-protein and quantification of unique peptides with parallel reaction monitoring.[10] After M-protein enrichment from the pre-ASCT serum sample and multienzymatic digestion, samples were analyzed with liquid chromatography with tandem mass spectrometry (*Online Supplementary Figure S2*). The full length M-protein sequence was assembled with the proprietary REmAb antibody sequencing platform. Unique tryptic peptides were identified after alignment to germline sequences and the LLOQ and LOD were ascertained for two to three peptides for individual patients. The diagnostic sample underwent serial dilution in the control serum, trypsin digestion, and analysis with parallel reaction monitoring assays. The LLOQ was the highest dilution at which the observed amount deviated from the expected amount by <20% and the coefficient of variation of duplicate injections was <20%. The clonotypic peptide with the lowest LLOQ was selected for tracking. To monitor an individual patient's M-protein, follow-up serum samples underwent digestion with trypsin and analysis with patient-specific parallel reaction monitoring assays to quantify the percent residual M-protein. Concordance between NGF and MS was assessed by the χ^2 and McNemar's tests.

A total of 62 patients were identified for analysis. MS analysis was successful in 57 (92%) patients. The five remaining patients all had light chain MM with involved FLC of 182-5,544 mg/L; four were sequenced but unique peptides could not be identified and one could not be sequenced due to polyclonal background and low protein level. In regard to the immunoglobulin isotype, 79% of patients had immunglobulin (Ig) G, 9% IgA, and 12% light chain MM. M-protein negativity by EasyM (MS^-) was achieved in only three (5%) patients, all of whom were enrolled on MM21. MS^- was first achieved at the pre-ASCT (light chain isotype), post-ASCT (IgG M-protein), and post-cycle 2 of consolidation (IgA M-protein) time points, respectively. All three patients remain in CR at 46-50 months post-ASCT. Sequential MS samples (Figure 1) revealed rising EasyM levels in six patients, coinciding with relapse in two patients and preceding relapse in four patients by 3, 15, 25,

and 38 months. Matched BM NGF results were available for five patients at time of rising EasyM levels, and of note, one patient was NGF MRD negative (NGF^-) whilst four patients were NGF MRD positive (NGF^+).

A total of 136 serum samples for MS with matched BM for NGF were available, with 25 pre-ASCT, 47 post-ASCT, 27 post-cycle 2 of consolidation, and 37 end of consolidation samples (Table 1). Forty-five samples (33%) were NGF^- compared to seven (5%) MS^- samples from three patients. MS^- was 4%, 4%, 7%, and 5% at the four time points. NGF^- was 8%, 23%, 37%, and 59% at the same time points. Ninety-eight (72%) samples were concordant. As patients progressed through treatment, the discordances between detectable M-protein by MS analysis and NGF^- (MS^+/NGF^-) increased, accounting for 4%, 19%, 30%, and 54% of samples at the four time points (Figure 2). McNemar's test *P* values were >0.999, 0.004, 0.008, and <0.001 for the four time points, indicating that MS^+/NGF^- were more likely than MS^-/NGF^+ discordances at all time points except for pre-ASCT. The χ^2 test for association between MS and NGF was not significant at any of the four time points. Of the 32 matched samples with confirmed serological CR status, nine (28%) were MS^+/NGF^+, 17 (53%) were MS^+/NGF^-, and six (19%) were MS^-/NGF^- ($\chi 2$ and McNemar's *P* values of 0.149 and <0.001). In this small cohort, MS^- was not significantly associated with progression-free survival.

This study compared sequential PB-based EasyM MS and BM-based EuroFlow NGF MRD in transplant-eligible MM patients enrolled on two clinical trials. The overall concordance rate between EasyM MS and EuroFlow NGF was 72%. This is comparable to the concordance rate of 78% between clonotypic MS and BM-based NGS MRD in patients enrolled on the IFM 2009 trial,[11] supporting the high sensitivity and reproducibility of MS despite differences in patient population and therapy. Furthermore, we demonstrated that 53% of CR samples were MS^+/NGF^-, consistent with the improved sensitivity of EasyM over BM-based evaluation with 8-colour multiparameter flow cytometry (MPFC) with sensitivity of 10^{-4}, a mix of

Table 1. McNemar's test of matched mass spectrometry (EasyM) and next-generation flow cytometry (EuroFlow platform) samples.

	NGF^+	NGF^-	McNemar's test H0: equal discordances (+/-=-/+)
All samples, N=136			
MS^+	91	38	Statistic: 36.026
MS^-	0	7	Exact probability: <0.001
Pre-ASCT, N=25			
MS^+	23	1	Statistic: 0
MS^-	0	1	Exact probability: >0.999
Post-ASCT, N=47			
MS^+	36	9	Statistic: 7.111
MS^-	0	2	Exact probability: 0.004
Post-cycle 2 of consolidation, N=27			
MS^+	17	8	Statistic: 6.125
MS^-	0	2	Exact probability: 0.008
End of consolidation, N=37			
MS^+	15	20	Statistic: 18.050
MS^-	0	2	Exact probability: <0.001
CR, N=32			
MS^+	9	17	Statistic: 15.059
MS^-	0	6	Exact probability: <0.001

NGF: next-generation flow cytometry; MS: mass spectrometry; ASCT: autologous stem cell transplantation; CR: complete response.

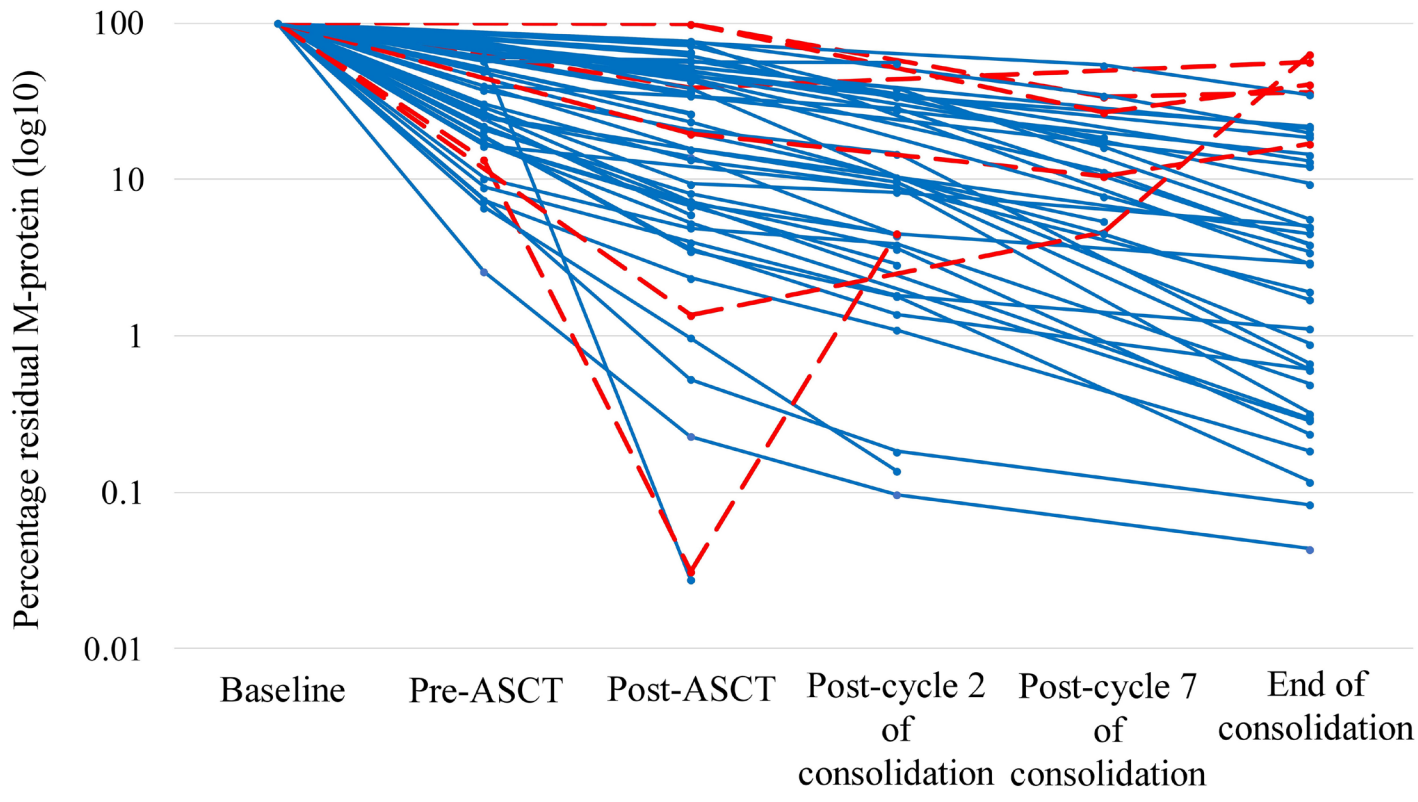

Figure 1. EasyM minimal residual disease kinetics in individual patients. Patients with rising EasyM are highlighted in red. ASCT: autologous stem cell transplantation; M-protein: monoclonal protein.

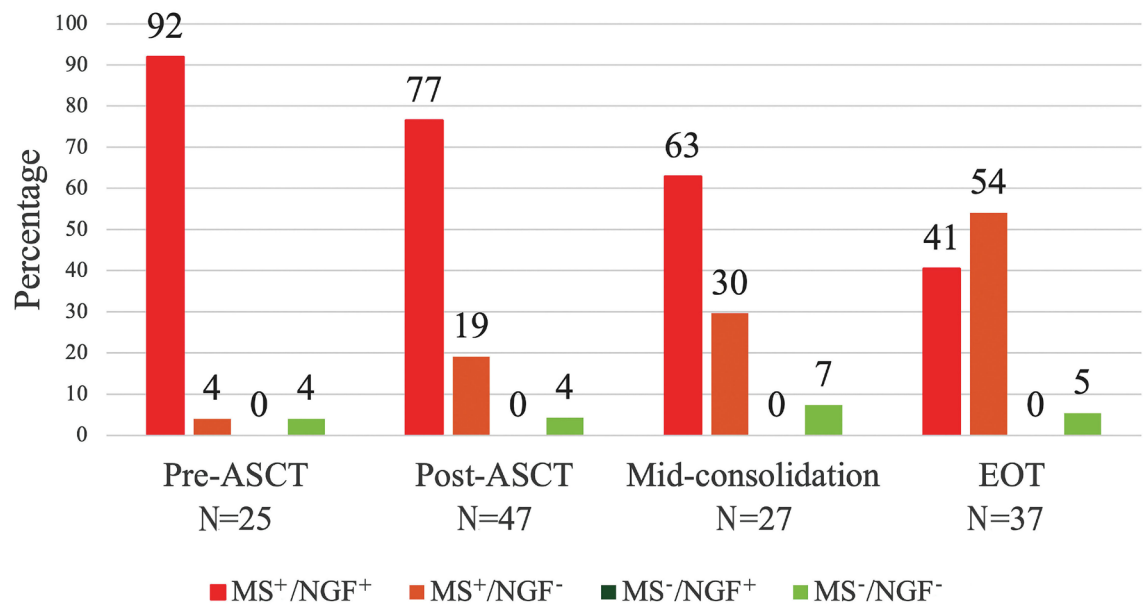

Figure 2. Matched multiple myeloma minimal residual disease assessment by mass spectrometry (EasyM) and next-generation flow cytometry (EuroFlow platform). ASCT: autologous stem cell transplantation; EOT: end of therapy; MS: mass spectrometry; NGF: next-generation flow cytometry.

MPFC and NGF with sensitivity of 10^{-4} to 10^{-5}, and clonoSEQ NGS with sensitivity of 10^{-5} to 10^{-6}.[10,12-13] These data suggest MS could guide appropriate timing and rationalize BM-based MRD testing in patients achieving CR, although the optimal cut-off values and timing are unknown. The ultrasensitive nature of EasyM rendered few samples MS^-. Studies have therefore calculated optimal MS^- cut-offs and demonstrated EasyM MS^+ predicts progression-free survival post-ASCT.[12-14] Prospective validation of clonotypic MS^- cut-offs and clinical outcomes is required.

Rising M-protein levels by MS predicted progression, detecting relapse up to 38 months earlier than traditional electrophoretic methods. Consistent with this, doubling M-protein levels by EasyM over 6 months predicted relapse.[10] Furthermore, retrospective data from IFM 2009 demonstrated that progression was detected with MS 442 days earlier on average,[11] underscoring the argument for dynamic MS MRD monitoring. All discordant results were MS^+/NGF^-, with an increasing proportion as patients progressed through treatment, suggesting deepening responses and increased MS sensitivity. Although achieving BM MRD negativity is a strong prognostic factor, patients with MRD negativity at a sensitivity of 10^{-6} continue to relapse over time, highlighting the unmet need for more accurate MRD detection.[15] False-negative NGF MRD results can be attributed to extramedullary disease, sampling error due to hemodilution, or the spatially heterogenous nature of BM infiltration characteristic of MM, arguing for PB-based approaches for M-protein detection. Additionally, concordance between MS and NGF could be affected by the different half-lives of M-proteins, especially in the early stages of monitoring IgG M-proteins which have extended half-lives due to neonatal Fc receptor recycling. EasyM cut-offs for different M-protein isotypes at day +100 post-ASCT have been proposed but require further evaluation.[14]

In conclusion, the EasyM PB clonotypic MS assay appears to be more sensitive in detecting residual disease in MM than the validated and widely accepted BM EuroFlow NGF MRD approach with a sensitivity of 10^{-5}. Concordance between MS and NGF was poor, with 53% of CR samples showing detectable M-protein by EasyM MS despite EuroFlow NGF MRD negativity. Comparison of larger sample sets and validation through prospective clinical trials is warranted to better assess the clinical utility and implications of EasyM MRD positivity. However, this preliminary data highlights the potential of EasyM for highly sensitive, sequential PB-based clonotypic MS MRD monitoring in MM, and consequently complementing MRD assessment in extramedullary MM, facilitating early relapse detection and adaptive response-based therapy, and rationalising BM-based MRD assessments.

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Contributions

AS designed the studies, performed research, analyzed data, wrote the initial draft and final draft, supervised the project. JZ annotated data, analyzed data, wrote the initial draft and final draft. TK, MG, AK, ZM, SM, NB, SL, DW, AJ, OM, NM, HQ, and LY performed research, approved the final draft. JR annotated data, analyzed data and approved the final draft.

Data-sharing statement

Data available on reasonable request to the corresponding author.

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