Circulating tumor DNA and bone marrow minimal residual disease negativity confers superior outcome for multiple myeloma patients

Multiple myeloma (MM) is a multi-focal genetically heterogeneous clonal plasma cell (PC) malignancy of the bone marrow (BM). BM-based minimal residual disease (MRD) assessment utilizing next generation flow (NGF - EuroFlow™) has become an important measure of treatment response and a validated predictor of outcome in MM, however, this approach and other single-site BM-derived assays may fail to capture the spatially heterogenous response to treatment evident in some patients. Analysis of blood-based circulating cell-free tumor DNA (ctDNA) has been shown to provide additional information to that provided by conventional disease assessment approaches.[1-5] In this study, we investigated if ctDNA molecular response could be utilized as an adjunct to MRD EuroFlow™ and the International Myeloma Working Group (IMWG) response criteria in predicting patient outcomes following secondary salvage therapy. We demonstrated that MM patients manifesting an early molecular response, as defined by a reduction in ctDNA burden, and who also achieved EuroFlow™ MRD negativity (MRD$^-$) had a superior outcome, providing the rationale to evaluate ctDNA in future studies as a non-invasive molecular response marker.

MM, a cancer of plasma cells, has a 5-year overall survival (OS) of 48.5% for newly diagnosed (ND) MM patients. Proteasome inhibitors and immunomodulatory drugs, with autologous stem cell transplantation (ASCT) have improved the survival rates, but patients eventually relapse due to the presence of remnant tumor cells (MRD+). While MM invariably relapses, MRD$^-$ patients consistently demonstrate more prolonged progression-free survival (PFS) and may represent a group where therapeutic de-escalation or modification can be safely considered.[6,7] However, despite this, over time an increasing proportion of MRD- patients relapse. In part, this may be because single-site BM biopsy-based MRD assessment does not capture the spatially heterogenous treatment response of MM patients, suggesting that alternative MRD approaches to identify responsive patients are necessary. Circulating ctDNA analysis is rapidly emerging as an adjunctive approach to single-site BM biopsy for genomic analysis, therapeutic monitoring and defining the underlying biology of resistance in MM.[8] Analysis of ctDNA addresses the spatial heterogeneity evident in MM and provides a more robust and risk-free methodology, thereby providing an alternative and a more practical modality to determine disease persistence. In this study, we investigated if alterations in the ctDNA mutational burden based on ultra-sensitive targeted amplicon sequencing (TAS) of 22 MM-relevant genes following treatment (molecular response) could be utilized as an adjunct to MRD EuroFlow™ and IMWG response criteria to improve the identification of primary refractory patients achieving the best response to secondary salvage therapy. The study population was from the Australasian Leukemia and Lymphoma Group (ALLG) MM17 phase II clinical trial of 50 transplant-eligible NDMM who were refractory, or had a sub-optimal response (SOR), to bortezomib-based first-line induction therapy (*ACTRN12615000934549*).[9] The trial evaluated an intensive salvage approach utilizing a combination of carfilzomib-thalidomide-dexamethasone (KTd) as re-induction (KTd x 4-6 cycles) and as ASCT consolidation (KTd x 2 cycles followed by Td x 10 cycles). Peripheral blood plasma in Streck BCT DNA tubes were obtained as per institutional ethics committee regulations and informed patient consent at study entry (baseline, N=48, 2 patients were excluded due to secondary malignancy and early death) and at cycle 3 day 1 (C3D1, N=46) (Figure 1). Plasma was processed for cell-free DNA as previously described.[10] Peripheral blood collected into EDTA tubes was utilized for *in vitro* isolation of peripheral blood mononuclear and genomic DNA extracted for use as germline control for TAS. A total of 142 samples (N=94 ctDNA and n=48 germline controls) was subject to TAS (Figure 1). One baseline sample failed sequencing and the final analysis cohort consisted of N=141 samples. Bioinformatic analysis was performed with QIAGEN's CLC Genomics Workbench using the HG38 human reference genome followed by QCI Interpret for variant calling.[11] Variant allele frequency (VAF), defined as the relative frequency of a mutated allele at a particular locus and expressed as a fraction or percentage of the overall allelic frequency (mutated + wild-type), was derived for each sample set. Single nucleotide variants (SNV) with a depth of coverage <10 in tumor or plasma samples and failed upstream filtering were excluded. The default filter settings on QCI Interpret for common genetic variants, predicting deleterious and cancer driver variants were employed. SNV and insertion/deletion polymorphisms (INDEL) appearing in the germline control were excluded utilizing the tumor-specific variants setting. Any variants that had an allele frequency of >=0.5% in at least one of the time points were included in the analysis.

Of the N=48 baseline samples, three samples did not have any baseline mutations detected and one sample failed sequencing, so were excluded from analysis (Figure 1). The VAF of mutations at baseline was utilized to calculate a fold change at C3D1. Three patients did not have C3D1 time points collected and were also excluded. Fold-change value was calculated as follows - if VAF of a specific baseline

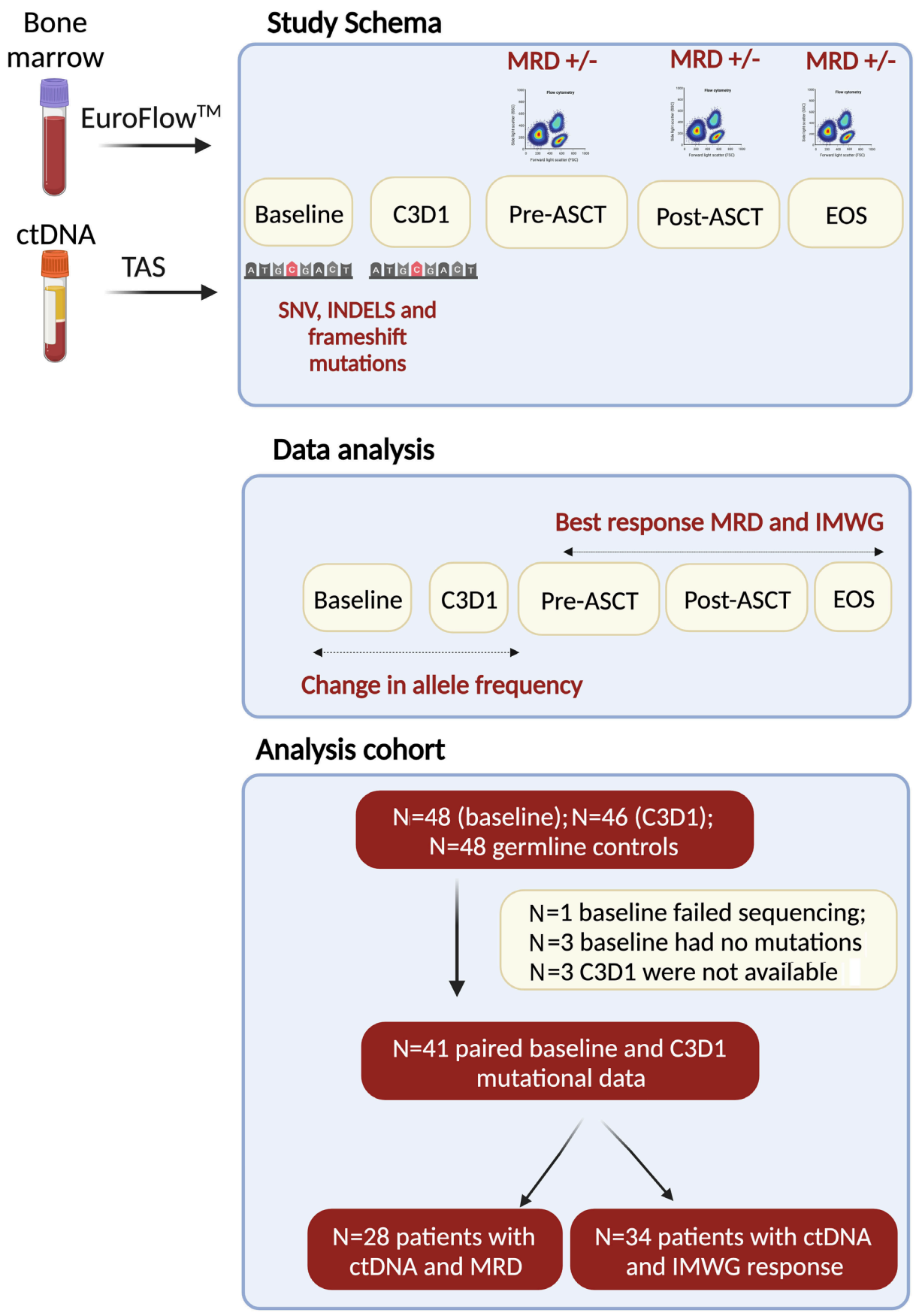

Figure 1. Study schema, data analysis and analysis cohort of the study. Study schema: peripheral blood samples from patients enrolled in the ALLG MM17 trial were collected as specific time points indicated. Single nucleotide variants (SNV), deletions, insertions (INDEL) and frameshift mutations were detected with targeted amplicon sequencing (TAS). Minimal residual disease (MRD) analysis was performed at pre-autologous stem cell transplantation (pre-ASCT), post-ASCT and post consolidation time points. Data analysis: International Myeloma Working Group (IMWG) response was defined pre-ASCT, post-ASCT and post-consolidation and the best response achieved along with best MRD response was recorded. The variant allele frequency (VAF) change between baseline and C3D1 samples was utilized to calculate a fold change in VAF with an average reduction as circulating cell-free tumor DNA (ctDNA$^-$) and an average increase as ctDNA$^+$. Analysis cohort: the number of samples utilized for TAS and correlation with IMWG response and/or MRD response is shown. BM: bone marrow; C3D1: cycle 3 day 1 of treatment; EOS: end of study. Figure generated with Biorender.com.

mutation is *x* and VAF of this mutation at C3D1 mutation is *y*, fold change is *(y-x)/x*. A negative fold change was defined as a decrease in VAF (ctDNA fold change negative or ctDNA$^-$) and a positive fold change an increase in VAF (ctDNA fold change positive or ctDNA$^+$) with the average fold change across all mutations calculated for each patient (Figure 1; *Online Supplementary Tables S1* and *S2*). EuroFlow MRD analyses were undertaken as previously described in adherence with the EuroFlow Consortium guidelines.[12] MRD analysis was performed pre-ASCT, post-ASCT and at the end of study (EOS) (Figure 1). A total of N=92 MRD assessments were available for analysis. The IMWG response was defined pre-ASCT, post-ASCT and post-consolidation. MRD and IMWG response for the purposes of outcome analyses was based on the best response achieved at any of the aforementioned time points (Figure 1; *Online Supplementary Table S1*). Seven patients had IMWG response criteria missing and out of these four patients had a relapse prior to the pre-ASCT assessment time point, two withdrew due to secondary malignancy and one had unevaluable serum measurements. Statistical analyses were performed using GraphPad Prism 9 (San Diego, CA, USA) and SAS 9.4 (SAS Institute, Inc., Cary, NC, USA). Progression-free survival (PFS) was measured from the date of commencing therapy to

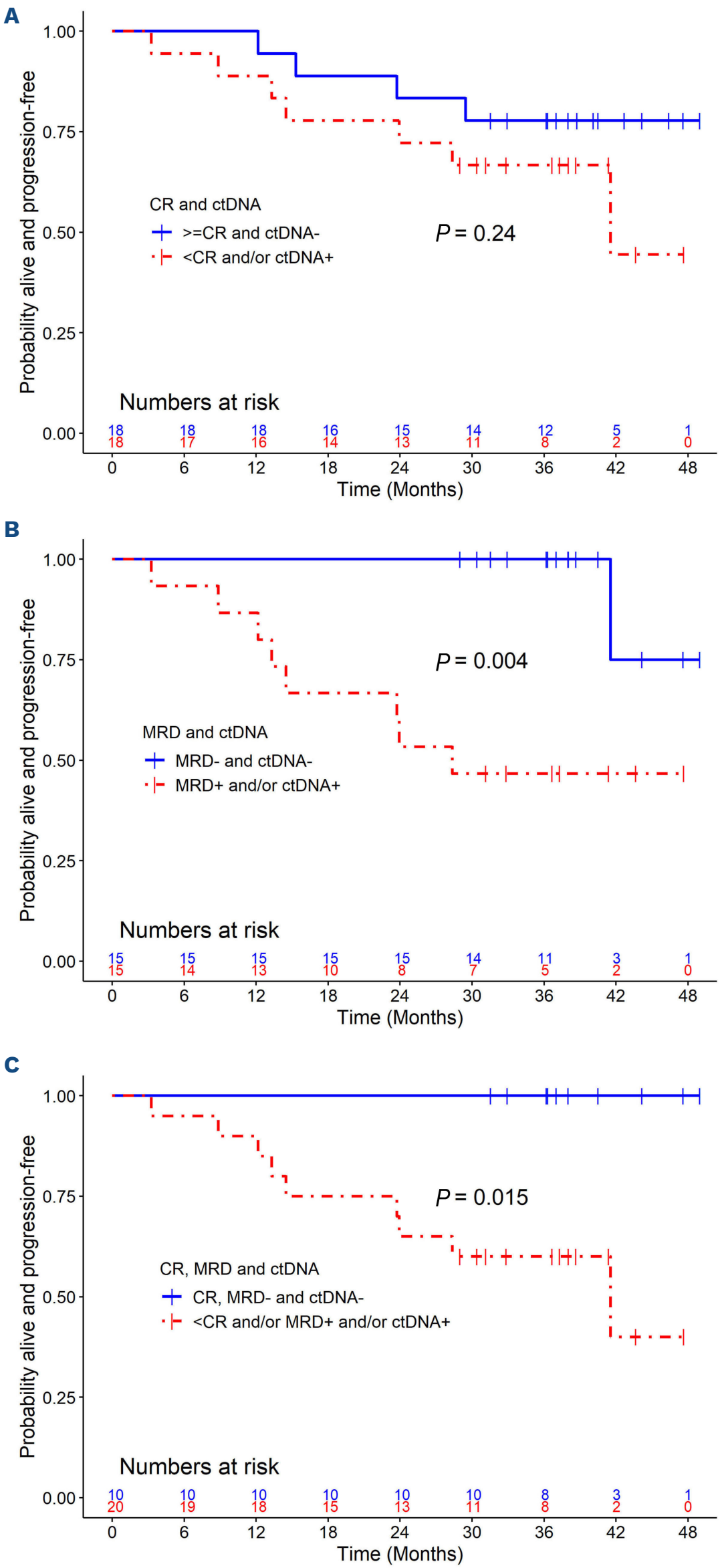

Figure 2. Patients with a circulating cell-free tumor DNA molecular response with minimal residual disease negativity and achieving complete response have a superior outcome. (A) Kaplan-Meier survival analysis for patients with ≥complete response (CR) and negative ctDNA$^-$ fold change demonstrated no significant differences in progression-free survival (PFS) when compared with patients with <CR and/or circulating cell-free tumor DNA increase or positive fold change from baseline (ctDNA$^+$) (*P*=0.24; log-rank test) (B) Kaplan-Meier survival analysis of patients grouped as negative minimal residual disease (MRD$^-$), reduction or negative fold change from baseline, and (ctDNA$^-$) (MRD$^-$ and ctDNA$^-$) or MRD$^+$ and/or ctDNA$^+$ demonstrated markedly superior PFS for patients achieving an early ctDNA molecular response and achieved MRD negativity (*P*=0.0040, MRD$^-$ ctDNA$^-$ (median PFS: not reached) *versus* MRD and/or ctDNA$^+$ (median PFS: 28.4 months). (C) Kaplan-Meier survival analysis combining International Myeloma Working Group response, MRD status and ctDNA response demonstrated that ≥CR, MRD$^-$ and ctDNA$^-$ patients had superior PFS compared to patients with <CR and/or were MRD$^+$ and/or ctDNA$^+$ (*P*=0.015; median PFS: not reached *vs*. 41.5 months).

the date of progression or death from any cause, whichever occurred first. Survival curves, with time in units of months, were plotted to investigate the association of PFS.

Of the N=48 baseline ctDNA samples, 44 patients had one or more mutations identified but of these three did not have a C3D1 time point. A total of 41 patients with complete TAS data were correlated with 'best' MRD status, IMWG response and PFS (Figure 1; *Online Supplementary Data 1*). A total of seven patients had a positive ctDNA fold change (i.e., ctDNA$^+$), while the rest had a ctDNA$^-$ fold change. Amongst the ctDNA$^+$ patients, two lacked MRD information and one lacked IMWG response. The remaining patients were not statistically large enough for further analyses. When patients with ≥CR and ctDNA$^-$ were compared with patients with <CR and/or ctDNA$^+$ no significant PFS difference was seen (P=0.24; log-rank test; Figure 2A). In contrast MRD$^-$/ctDNA$^-$ patients demonstrated markedly superior PFS when compared to MRD$^+$ and/or ctDNA$^+$ patients, not reached *versus* 28.4 months (P=0.004), respectively (Figure 2B). Finally combining all three response categories demonstrated that patients who were ≥CR, MRD$^-$ and ctDNA$^-$ also had a superior PFS compared to patients who were <CR and/or MRD$^+$ and/or ctDNA$^+$, not reached *versus* 41.5 months (P=0.015), respectively (Figure 2C). Our results demonstrate that treated MM patients manifesting an early molecular response, as defined by a reduction in ctDNA mutational burden by C3D1, and who also achieve MRD negativity have a superior outcome. These results demonstrate the utility of ctDNA-based early molecular response as a predictor of patient outcome in MM.

Our study employs Streck tubes that prevent cell rupture, ensuring contamination-free ctDNA collection. These tubes are compatible with multi-center studies, stable for 72 hours at ambient temperature, and consistently yield high-quality ctDNA for TAS when following the manufacturer's recommended standard operating procedures. The ultra-sensitive ctDNA mutational analysis method that we have developed offers real-time insight into minute VAF alterations. Results are available within 1 week of blood collection and the approach sheds light on disease biology as specific mutations are linked to disease progression. In contrast, alternative blood-based methods like circulating tumor cells require a fresh blood sample, intricate enrichment techniques and might not be readily applicable for multi-center studies.[13] Similarly, serum based matrix-assisted laser desorption/Ionization time-of-flight or clonotypic peptide approach could be potentially useful for MRD detection.[14] However, these techniques do not capture the biological changes that occur in response to treatment or disease progression. The limitations of our study are the small sample size mandating that larger confirmatory studies be undertaken to validate these observations. If validated, ctDNA would represent a readily accessible analyte for outcome prediction, thus facilitating early intervention and response adaption in MM patients destined to fail treatment.

Authors

Sridurga Mithraprabhu,[1,2] John Reynolds,[1,2] Hang Quach,[3] Noemi Horvath,[4] Ian Kerridge,[5] Tiffany Khong,[1,2] Brian GM Durie[6] and Andrew Spencer[1,2]

[1]Australian Center for Blood Diseases, Alfred Health - Monash University, Melbourne, Victoria, Australia; [2]Department of Malignant Hematology and Stem Cell Transplantation, Alfred Hospital, Melbourne, Victoria, Australia; [3]St.Vincent's Hospital, University of Melbourne, Melbourne, Victoria, Australia; [4]Royal Adelaide Hospital, Haematology, Adelaide, South Australia, Australia; [5]Royal North Shore Hospital, Haematology, Sydney, New South Wales, Australia and [6]Cedars-Sinai Comprehensive Cancer Center, Los Angeles, CA, USA

Correspondence:
A. SPENCER - Andrew.spencer@monash.edu
S. MITHRAPRABHU - Durga.mithraprabhu@monash.edu

https://doi.org/10.3324/haematol.2023.283831

Received: June 25, 2023.
Accepted: September 19, 2023.
Early view: September 28, 2023.

Disclosures

No conflicts of interest to disclose.

Contributions

SM designed the study, performed experiments, analyzed and interpreted data and wrote the manuscript. JR performed statistics and data interpretation. HQ, NH, IK and TK collected samples and clinical data. BD interpreted the data and contributed to presentation. AS designed and established the study, collected samples and clinical data, interpreted data and wrote the manuscript. All authors contributed to editing and approving the manuscript.

Acknowledgments

The authors would like to acknowledge the contribution of the staff and patients at the Malignant Hematology and Stem Cell Transplantation, Alfred Hospital, the staff at the Department of Pathology, Alfred Hospital and the Australasian Leukemia and Lymphoma Group (ALLG) network.

Funding

This study was funded by the International Myeloma Foundation's Black Swan Research Initiative and philanthropic funding through the Alfred Foundation.

Data-sharing statement

Data will be made available on reasonable request addressed to the corresponding authors.

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