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## Cell-free DNA for the detection of emerging treatment failure in relapsed/refractory multiple myeloma

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## Abstract

Interrogation of cell-free DNA (cfDNA) represents an emerging approach to non-invasively estimate disease burden in multiple myeloma (MM). Here, we examined low-pass whole genome sequencing (LPWGS) of cfDNA for its predictive value in relapsed/refractory MM (RRMM). We observed that cfDNA positivity, defined as  $\geq 10\%$  tumor fraction by LPWGS, was associated with significantly shorter progression-free survival (PFS) in an exploratory test cohort of 16 patients who were actively treated on diverse regimens. We prospectively determined the predictive value of cfDNA in 86 samples from 45 RRMM patients treated with elotuzumab, pomalidomide, bortezomib and dexamethasone in a phase II clinical trial (*NCT02718833*). PFS in patients with tumor-positive and -negative cfDNA after two cycles of treatment was 1.6 and 17.6 months, respectively (HR 7.6,  $P < 0.0001$ ). Multivariate hazard modelling confirmed cfDNA as independent risk factor (HR 96.6,  $P = 6.92 \times 10^{-5}$ ). While correlating with serum-free light chains and bone marrow, cfDNA additionally discriminated patients with poor PFS among those with the same response by IMWG criteria. In summary, detectability of MM-derived cfDNA, as a measure of substantial tumor burden with therapy, independently predicts poor PFS and may provide refinement for standard-of-care response parameters to identify patients with poor response to treatment earlier than is currently feasible.

## Keywords

cell-free DNA; circulating tumor DNA; liquid biopsy; next-generation sequencing; precision medicine

## Introduction

Multiple myeloma (MM) is a hematologic plasma cell malignancy with multifocal dissemination throughout the bone marrow (BM). Despite continued and considerable progress over the past decade, MM remains an incurable disease which ultimately develops drug resistance and evades treatment<sup>1</sup>. Resistant disease is typically driven by a high degree of genomic heterogeneity which causes some patients to benefit more from a certain therapy than others<sup>2-7</sup>. With each relapse, treatment regimens are switched, but remissions tend to get shorter in duration with each new regimen<sup>8,9</sup>. With a growing number of available treatment options, efforts to monitor disease activity repeatedly over time become increasingly important. Ideally, such improved monitoring will help identify early signs of disease recurrence before patients experience symptoms from overt relapse or refractory disease.

Current therapeutic monitoring of MM relies on PET/CT imaging, serum biomarkers and serial single-site BM biopsies<sup>10</sup>, which however may fail to capture the intrinsic spatial and temporal heterogeneity of MM in some patients<sup>11,12</sup>. This is particularly relevant for patients with extramedullary disease (EM-MM) or patients who lack conventional biomarkers, such as those with non-secretory MM, for whom imaging and BM biopsies remain the only modalities available for therapeutic monitoring<sup>13-15</sup>.

Over the past years, liquid biopsy technologies, i.e. the interrogation of circulating MM cells (CMMCs) or nucleic acids from the blood or body fluids, have emerged as a promising minimally invasive hallmark for the diagnostic management of MM<sup>16</sup>. Cell-free DNA (cfDNA) represents one potential source for such liquid biopsy profiling as it is shed into circulation through apoptosis and necrosis of tumor cells<sup>17–19</sup>. Given its rapid clearance from the bloodstream (half-life ~1.5 h), it can provide insight into changes of tumor burden and clonal evolution *in real-time*<sup>20–22</sup>.

Different methodologies with varying cost, sensitivity and applicability have been proposed for the assessment of cfDNA. Current technologies range from unbiased, discovery-oriented approaches such as whole genome sequencing (WGS) and/or whole exome sequencing (WES)<sup>23,24</sup> to low-pass WGS (LPWGS) as an emerging cost-efficient technology to measure tumor burden by estimation of copy number variants (CNV)<sup>17</sup>. Targeted approaches together with PCR-based technologies exploit prior knowledge of mutations to perform deep DNA sequencing of somatic mutations<sup>25–30</sup> or clonally rearranged IGH genes<sup>31–33</sup>. With the entirety of these methods it is not only possible to reproduce the mutational landscape in the BM but to also detect cfDNA-exclusive mutations, thereby overcoming the spatial sampling bias that is inherent to BM biopsies<sup>27,28</sup>. Several studies have investigated the clinical value of cfDNA sequencing in the context of high-risk disease and prognostic outcome. Available data demonstrates correlation of cfDNA with serum biomarkers as well as with disease stages, with cfDNA levels being lowest in precursor states and highest in patients with RRMM<sup>23,24</sup>. The added value for clinical decision-making in MM however remains unclear as sample sizes and treatment homogeneity have remained limited in prior analyses<sup>25,26,29,32,34–36</sup>.

The objective of our study was to determine the predictive value of cfDNA interrogation by LPWGS for patients with RRMM. We investigated if cfDNA can identify patients with emerging drug resistance. We postulated that the kinetics of MM-derived cfDNA in response to treatment distinguish between patients with good and poor outcomes. Having such orthogonal predictive markers at hand has the potential to motivate changes in treatment at an early stage before relapse emerges by currently established clinical parameters.

## Materials/Subjects and Methods

### Patients and Sample Collection

Samples for our exploratory test cohort were acquired from patients at remission stage or while undergoing induction therapy (n=16, Table 1). Samples for our validation cohort were acquired from 45 patients (Table S1) treated within a multi-center phase II trial testing elotuzumab with pomalidomide-bortezomib-dexamethasone (elo-PVD) for RRMM<sup>37</sup>. All samples for this portion of the trial were retrieved at screening and at cycle 3 day 1 (C3D1) of elo-PVD. 44 and 42 samples were available for analysis at screening and C3D1, respectively, for a total of 86 samples (Figure 1A). In brief, elotuzumab was administered by IV infusion on a 28-day cycle starting with weekly administration (cycle 1–2), followed by bi-weekly infusion (cycle 3–8) and ultimately administration every four weeks (cycle 9+). Pomalidomide was given PO on day 1–21, bortezomib was injected SC on day 1, 8 and 15 of each cycle and dexamethasone was added weekly. Treatment continued until progression,

unacceptable toxicity, patient refusal or changes in the patient's condition that prohibited further treatment according to investigator decision. A detailed summary of screen failure patients, patients with serious adverse event and sample dropouts is given in Table S1.

All patients provided written informed consent to allow for the collection and research analysis of blood and BM under DFCI protocol #19–511 (test cohort) and DFCI protocol #15–475 (validation cohort). The entire study was performed in accordance with the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practice.

### Plasma separation and cfDNA extraction

Deidentified blood samples were drawn from eligible patients in EDTA tubes (Becton Dickinson, Franklin Lakes, NJ), transported on ice and processed within 3 hours. Plasma was isolated by centrifugation of whole blood at 1500 rpm for 10 min at 4°C. The clear plasma was transferred to a fresh tube and centrifuged a second time at 3500 rpm for 10 min at 4°C to remove residual cells. Supernatants were then frozen in 1 ml aliquots (Corning Inc., Corning, NY) at –80°C until further processing. Frozen aliquots of plasma were thawed on ice. CfDNA was extracted from 1 ml of plasma using the Quick-cell-free DNA Serum & Plasma Kit (Catalog #D4076, Lot #ZRC187039, Zymo Research, Irvine, CA) and was stored at –20°C until further processing. Quantification of cfDNA was performed using the Qubit dsDNA HS assay kit (ThermoFisher Scientific, Waltham, MA).

### Library construction and sequencing of cfDNA

An input of 10 ng of cfDNA was used for LPWGS with sequencing adapters as described<sup>24</sup>. Enrichment of adapter-ligated libraries was generated by amplification of adapter-ligated DNA with Ultra II Hotstart PCR Master Mix (Agilent Technologies, Santa Clara, CA). Size selection was performed using Agencourt Ampure XP beads (#A63881, Beckman Coulter, Brea, CA) and DNA was quantified using the Qubit dsDNA HS assay kit (ThermoFisher Scientific, Waltham, MA). Samples were pooled assuming an average fragment size of 300 bp, and quality was verified on the 2200 TapeStation using a High Sensitivity D1000 ScreenTape (Agilent Technologies, Santa Clara, CA). A normalized amount of 1.8 pM of denatured library was sequenced on the NextSeq500 (Illumina, San Diego, CA) at an average depth of 0.22X. Paired-end, 37-bp reads were generated using a High Output, 75 cycle kit with v2 chemistry (Illumina, San Diego, CA). Reads were aligned to the hg19 reference human genome by BWA (version 0.7.13) with default parameters. Duplicated reads were filtered out using Picard (<https://broadinstitute.github.io/picard/>).

### ichorCNA analysis and ctDNA quantification

The MM-specific tumor fraction in total cfDNA was determined using *ichorCNA* as previously published<sup>17</sup>, by computing read coverage and predicting large-scale CNV and tumor fractions without knowledge of prior mutations (Figure 1A). *ichorCNA* is implemented as an R package and can be obtained at <https://github.com/broadinstitute/ichorCNA>. Low coverage samples were additionally reviewed manually by two blinded investigators for accurate tumor fraction estimation.

## Statistical Analysis

The median length of follow-up in our validation cohort was 29.7 months. The primary objective of this study was to evaluate the detectability of cfDNA tumor fractions in a uniform cohort of RRMM patients treated with elo-PVD. The secondary objective was to investigate cfDNA assessment at C3D1 as an early indicator of outcome in comparison to response as defined by the International Myeloma Working Group (IMWG) guidelines for the same time point. Calculations of median PFS were performed by Kaplan-Meier estimators using the *survival* (version 2.44) and *survminer* (version 4.6) packages from R (version 3.6.1). P values for all PFS comparisons were calculated with the log-rank (Mantel-Cox) test. Statistical analysis was performed using the Mann-Whitney t-test for unpaired samples and Fisher exact test for categorical variables. A P value of  $P < 0.05$  was considered to be statistically significant and levels of significance were marked as follows:  $P < 0.05$  (\*),  $P < 0.01$  (\*\*), and  $P < 0.001$  (\*\*\*). Cox proportionality hazard models for the prediction of PFS at baseline and at C3D1 of treatment were applied by integrating relevant covariates using *survminer* (version 0.4.6) from R. For all assessments involving cfDNA measurements at C3D1, PFS was defined as time calculated from C3D1 to clinical progression (landmark analysis)<sup>38</sup>. Forest plots were generated based on Cox model analysis to determine the predictive value of cfDNA tumor fraction thresholds on PFS independent of IMWG response and other clinical covariates. Schoenfeld residuals were calculated to test the proportional hazards assumption, with  $P > 0.05$  indicating non-violation of the proportional hazard assumption.

## Results

### MM-derived cfDNA as proxy for tumor burden and predictor of progression-free survival

Our first goal was to gather evidence that persistence of cfDNA in patients undergoing active treatment is associated with worse outcome. To this end, we investigated a test cohort of 16 MM patients who were actively receiving a variety of treatments and compared PFS between patients with and without detectable MM-derived cfDNA (Figure 1A, Table 1). We employed our recently described LPWGS-based approach<sup>24</sup>. Copy number alterations and tumor fractions from cfDNA were predicted using the *ichorCNA* algorithm (Figure 1A). Positivity in cfDNA samples was defined by applying a threshold of 10% which has previously been defined as reproducible and robust for the context of LPWGS data<sup>23,24</sup>.

In this cohort with heterogeneous treatments and limited sample size (Table 1), 3/16 patients were tested positive and 13/16 were tested negative for the presence of MM in cfDNA. Patients with detectable tumor fraction experienced a significantly shorter PFS (0.7 vs. 32.7 months, hazard ratio 9.9 (0.4 to 238),  $P < 0.0001$ , Figure 1B) suggesting higher tumor burden in those patients. To explore if an increase in MM-derived cfDNA might also be a direct consequence of treatment and thus reflects tumor killing, we closely examined the kinetics of cfDNA tumor fractions by weekly monitoring of cfDNA in two patients: Pt01T with intermittently decreasing and Pt02T with stable course of their involved serum-free light chains (iFLC, Figure 1C,D). Both patients had a short PFS of 2.8 and 2.4 months, respectively, and in both patients cfDNA samples were constantly positive for residual MM disease until the end of treatment. Importantly, in both patients, even as early as one week

after initiation of treatment, we observed a modest decline in cfDNA tumor burden, rather than a treatment-related spike, indicating that cfDNA is a robust reflection of tumor burden even for patients who actively receive treatment.

Based on this preliminary observation in a small exploratory test cohort, we next sought to test the following hypotheses: 1) The presence of detectable MM-derived cfDNA is predictive of PFS when determined in the early course of therapy, 2) MM-derived cfDNA correlates with measures of clinical response and disease burden used in the clinical routine, but in addition identifies patients who experience only very short PFS and who might therefore be candidates for early therapeutic intervention before relapse is detectable by clinical routine parameters. To test these hypotheses and reduce treatment bias as a confounding variable, we identified a controlled cohort of 45 RRMM patients who had been uniformly treated in a multicenter phase II trial of elotuzumab, pomalidomide, bortezomib and dexamethasone (elo-PVD, [clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02718833) identifier: NCT02718833) (Figure 1A).

We investigated 44 and 42 available cfDNA samples for time of screening and C3D1 of elo-PVD, respectively. 14/44 (32%) screening samples were found to be positive. At C3D1, 11/42 (26%) samples had positive cfDNA values. Four patients with positive cfDNA at screening tested negative at C3D1, whereas two patients with negative cfDNA at screening showed positive cfDNA results at C3D1. At time of data analysis, 9/45 (20%) patients remained on trial, while 36/45 (80%) patients had already relapsed. Clinical characteristics are detailed in Table 2, Table S1, Table S2 and Table S3.

At screening, MM positivity by cfDNA was associated with a short PFS of 3.7 months as compared to a PFS of 20.1 months in patients with tumor-negative cfDNA samples (hazard ratio 4.5 (1.7 to 12),  $P < 0.0001$ , Figure 2A, Table S2). Interestingly, patients with cfDNA-positive vs. -negative samples at screening showed comparable ISS stage ( $P > 0.999$ ), high-risk cytogenetics ( $P > 0.999$ , defined as del17p, t(4;14), t(14;16) and/or t(14;20)), iFLC values ( $P = 0.644$ ), serum M protein ( $P = 0.503$ ) and BM infiltration ( $P = 0.323$ ) (Table S2, Figure S1). However, patients with cfDNA-positive samples had been exposed to a significantly higher number of prior therapies ( $P = 0.047$ ), were older ( $P = 0.033$ ) and had significantly higher LDH serum levels at baseline ( $P = 0.0004$ ) than patients with negative test result. 3/14 (21%) patients with positive cfDNA had evidence of extramedullary MM (EM-MM), whereas no cfDNA-negative patient was determined to have EM-MM ( $P < 0.0001$ ).

Next, we evaluated patients with available follow-up samples ( $n = 42$ , Table S1) for their tumor fractions at C3D1 of elo-PVD to determine if MM persistence in cfDNA was associated with poor PFS. Median tumor fraction in cfDNA of C3D1 samples was 3% (0–47%). A tumor fraction  $< 10\%$  was associated with a significantly longer PFS (median 17.6 months) as compared to residual positivity in cfDNA (median PFS 1.6 months, hazard ratio 7.6 (1.8 to 31.3),  $P < 0.0001$ , Figure 2B, Table 2). Patients with positive vs. negative cfDNA at C3D1 showed no significant difference in ISS stage ( $P = 0.243$ ), high-risk cytogenetics ( $P = 0.283$ ), evidence of EM-MM ( $P = 0.163$ ) or number of prior lines of therapy ( $P = 0.294$ ). While serum M protein ( $P = 0.525$ ) and LDH levels ( $P = 0.457$ ) at C3D1 neither revealed significant difference for both groups, we observed significantly increased serum iFLC levels ( $P < 0.0001$ ) and BM infiltration at C3D1 ( $P = 0.024$ ) in patients with



positive vs. negative cfDNA sample at C3D1 (Table 2). These data demonstrate that residual detectability of MM-derived cfDNA after two cycles of elo-PVD is associated with poor PFS.

### Correlation of cfDNA with BM infiltration and serological parameters

We next determined the correlation of MM-derived cfDNA with established markers of MM burden in those samples with detectable tumor fraction in cfDNA  $\geq 10\%$ . Cell-free DNA showed significant correlation with iFLC levels (Figure 3A, Spearman 0.64 (95%CI 0.28 to 0.84),  $P=0.001$ ), indicating a close relation between both biomarkers. Patients with residual cfDNA-positive disease at C3D1 also showed significantly increased iFLC levels (median 594 mg/L, range 22–5012 mg/L, Table 2) as compared to patients with no detectable tumor fraction at C3D1 (median 46, range 1–1670 mg/L,  $P=0.004$ ). While correlation with BM infiltration in paired plasma samples showed a trend toward positive correlation (Figure 3B, Spearman 0.39 (95%CI  $-0.2$  to 0.77),  $P=0.171$ ), this comparison did not reach significance, most likely due to two outlier patients (Pt01V, Pt02V) with high tumor burden measured in cfDNA but low BM infiltration (Figure 3C). Interestingly, high cfDNA tumor burden translated into a very short PFS on elo-PVD (3.8 and 1.2 months) in these patients (Figure 3C). This indicates that cfDNA may be a valuable resource to identify patients with high tumor burden in whom BM infiltration is falsely low due to technical and/or spatial bias of BM biopsies.

### Refining IMWG response parameters with cfDNA

Next, we compared tumor fraction response in cfDNA after two months of elo-PVD with the depth of response as defined per standard of care. To this end, we determined tumor fractions at first follow-up (C3D1) as a function of depth of response by IMWG guidelines<sup>39</sup>. At C3D1, 3/42 (7%) patients had already progressed, 20/42 (48%) patients sustained stable disease (SD), 13/42 (31%) patients had partial remission (PR) and 6/42 (14%) patients had achieved very good partial remission (VGPR). As expected, patients with PD had the highest mean tumor fraction at C3D1 (21%, range 17–28%), followed by patients with SD (7%, range 0–38%), PR (6%, range 0–47%) and VGPR (4%, range 0–11%). Notably, we observed considerable variation of individual cfDNA tumor fractions within the subgroups of patients with SD, PR and VGPR after two cycles of elo-PVD, including several outliers with very high tumor fractions despite apparent response according to IMWG criteria (Figure S2).

To further test our hypothesis that cfDNA can discriminate between patients with good and poor PFS despite identical IMWG-defined depth of response, we performed a subgroup survival analysis (Figure 4A). In the subgroup of SD patients at C3D1 ( $n=20$ ), our approach segregated patients into cfDNA-positive patients with a poor prognosis (median PFS 1.5 months) and cfDNA-negative patients with a more favorable outcome (median PFS 6.8 months, hazard ratio 7.2 (95%CI 0.9 to 59),  $P<0.0001$ , Figure 4B). For patients with a PR at C3D1 ( $n=13$ ), the same threshold defined cfDNA-positive patients with an inferior outcome (median PFS 2.2 months) and cfDNA-negative patients with a more favorable prognosis (median PFS 12.1 months, hazard ratio 6.1 (95%CI 0.3 to 142),  $P=0.007$ , Figure 4B).

These data demonstrate that detectability of residual cfDNA tumor fractions after treatment initiation identifies patients with imminent relapse and poor PFS.

Examples for which cfDNA may be able to serve as an orthogonal marker to refine serological assessment are shown in Figure 4C and 4D. Pt03V who was assessed to have SD after two cycles of elo-PVD, experienced an increase in cfDNA tumor fraction from 11% to 17% and relapsed shortly afterwards (PFS 3.3 months) despite a decline in serum M protein at C3D1 (Figure 4C). Pt04V was classified as having achieved a PR after two months of elo-PVD but sustained high levels of tumor fraction and relapsed after 4.3 months on the trial, despite a decline in his iFLC levels at C3D1 (Figure 4D). Cell-free DNA may therefore provide the opportunity to refine IMWG response criteria as an independent marker of response.

### **Persistent cfDNA tumor fraction after two cycles of elo-PVD as independent prognostic risk factor**

To formally define cfDNA as a predictive marker independent of established parameters of response, we calculated multivariable Cox regression and estimated hazard ratios for associations between PFS on elo-PVD treatment and i) residual MM-derived tumor fraction in cfDNA at C3D1, and ii) IMWG response at C3D1. In this multivariate analysis, residual positivity for MM in cfDNA at C3D1 was confirmed as a significant and independent prognostic factor that correlated with inferior outcome (hazard ratio=96.6,  $P=6.92e-05$ , Figure 5, Figure S3). Detectable MM-derived cfDNA at screening also conferred shorter PFS, although less significantly (hazard ratio 4.6,  $P=0.0005$ , Figure S4). Neither the achievement of VGPR ( $P=0.646$ ) nor PR ( $P=0.315$ ) reached a comparable prognostic value. Interestingly, other markers that are well established to predict unfavorable outcome when obtained at diagnosis, i.e., age >50 years, 1 prior line of treatment, ISS stage III and high-risk cytogenetics did not demonstrate independent prognostic value. These data propose cfDNA as an independent predictive marker, which may be of particular value in patients with multiple prior treatments.

## **Discussion**

Based on our findings in a small test cohort of MM patients with heterogeneous anti-MM treatments that residual detectability of MM-derived cfDNA is associated with poor PFS, we hypothesized that estimation of MM-derived tumor fraction in cfDNA by LPWGS can be used as a predictive marker for disease progression and therapy response in an independent, uniformly treated cohort of patients with RRMM.

We demonstrate that persistence of MM-specific cfDNA after two cycles of elo-PVD is a poor-prognosis marker that is associated with short PFS. We show that while monitoring of disease burden by cfDNA is generally concordant with IMWG criteria, it provides added utility over IMWG criteria for detecting emerging resistant disease. We also report patients with short PFS and high tumor burden by cfDNA despite discrepantly low BM infiltration, supporting the notion that cfDNA is less susceptible to spatial and technical bias than a BM biopsy.



Our findings indicate that determination of MM-specific cfDNA by LPWGS can help with early detection of imminent relapse independent of serological parameters. After two months of therapy, the detection of residual MM-derived cfDNA was associated with a significantly shorter PFS. Strong concordance was noted between detectable tumor fractions and IMWG responses in the majority of patients. However, we identified remarkable discrepancy in the subgroups of patients classified as SD and PR according to IMWG response criteria after two months of therapy. The prognosis of patients with SD worsened from a median PFS of 6.8 to 1.5 months if the follow-up sample after two cycles of elo-PVD was tested positive for residual MM tumor burden. Similarly, patients with a PR faced a median PFS of 2.2 instead of 12.1 months if they were confirmed to have MM-positive cfDNA at C3D1. These data demonstrate that persistent tumor fractions in cfDNA can help identify patients who do not benefit from a particular treatment regimen when currently available biomarkers remain unchanged. In this context, cfDNA may represent a more dynamic measure for the tracking of MM since it may be cleared from the peripheral blood at a higher rate than serum M protein or iFLC <sup>15</sup>.

While our study is the first to demonstrate independent predictive value of cfDNA, more work is needed to evaluate if early detection of relapse can be translated into improved outcomes for patients. Our independent test cohort of heterogeneous MM patients together with our validation cohort suggest that the cfDNA approach that we propose represents a robust predictive tool across different treatment scenarios in RRMM.

In our study we focused on cfDNA evaluation after two cycles of therapy as a practical and cost-effective approach. We advocate for including serial cfDNA monitoring in the context of multi-center trials to inform response-adapted treatment strategies in *real-time* and to determine if our findings are agnostic of the specific treatment. We expect that higher efficacy of treatment regimens would further increase the power of cfDNA as a dynamic prognostic tool. Since CNV are detectable in the overwhelming majority of MM patients <sup>40,41</sup>, LPWGS enables estimation of tumor fractions in the majority of patients with relapsed disease <sup>23,24</sup>. This unbiased methodology is highly scalable, cost-effective, can be completed with a short turnaround time and does not require prior knowledge of the individual mutational profile of a patient. It is therefore particularly useful in the advanced setting of RRMM when tumor burden is high and molecular as well as spatial heterogeneity of the disease are actively evolving.

In line with prior reports <sup>23,24</sup>, we here evaluated the predictive value of a tumor fraction threshold in cfDNA of 10%. Over time and with changing treatment regimens MM cells undergo considerable changes, which affect their transcriptome, proteome including surface markers or metabolism <sup>42,43</sup>. Such changes may affect their secretion of iFLC, avidity for PET tracers or their overall fragility. More fragile cells may spontaneously lyse at a higher rate resulting in a higher cfDNA tumor fraction. We therefore chose the robust cutoff of 10% tumor fraction for our study to avoid overinterpretation of small relative changes in cfDNA and mitigate these sources of variation. Robust detection of tumor fractions <10% or less is possible with our methodology by increasing sequencing depth. In this scenario, targeted sequencing approaches however are more cost-effective and may be more suitable to detect somatic mutations that have previously been identified <sup>26–28,33,35,44</sup>.

One might hypothesize that certain patient subpopulations would especially benefit from complementary cfDNA assessment. For patients with EM-MM, molecular monitoring is challenging either due to the inaccessibility of lesions in the body or the inability to perform invasive sampling repeatedly over time. In an elegant study by Mithraprabhu and colleagues driver mutations were constantly tracked in cfDNA from EM-MM patients and increased over the disease course, whereas serological parameters falsely indicated disease control<sup>35</sup>. Our validation cohort included 3/45 patients (7%) with EM-MM. Cell-free DNA tumor fractions for these patients at study entry varied between 14% and 33%. In two patients, cfDNA samples remained positive at C3D1 which translated into an unfavorable PFS of 2.8 and 3.8 months. The third patient had a tumor fraction response (TF<10% in cfDNA at C3D1) which was associated with a more favorable PFS of 10.0 months. In addition, correlation with paired BM samples identified two outlier patients with high cfDNA tumor fraction and short PFS on elo-PVD despite low BM infiltration at baseline. These data support that single-site biopsies and serological markers cannot always serve as a reliable genomic representation of the multifocal nature of MM and that cfDNA sequencing may provide a more comprehensive approach to monitor patients for whom standard monitoring is not available. This may also apply to patients with non-secretory disease<sup>13,14</sup> and to elderly and frail MM patients with significant comorbidities, who are less likely to be subjected to frequent BM biopsies<sup>45</sup>. For these patients, cfDNA sequencing is attractive, since the technology allows for shipping from remote locations at room temperature in preservative-containing tubes, as means to obtain comprehensive molecular information without the need for more invasive biopsies<sup>46,47</sup>.

Liquid biopsy approaches in MM are not limited to cfDNA. Several other methodologies have been described, including the querying of molecular information from i) CMMCs<sup>23,48–56</sup>, ii) cell-free RNA<sup>27</sup> and iii) microRNAs<sup>48–50</sup>. CMMCs have procedural advantages over cfDNA since deep sequencing can be integrated with single-cell NGS and phenotypic characterization by next-generation flow (NGF). However, this limitation may be less relevant in the routine since MM is a systemic disease in most cases and effective therapeutic concepts need to address all existing genetic alterations. More work is required to determine the circumstances in which these complementary methods show the greatest clinical benefit.

In summary, our work suggests that LPWGS of cfDNA provides information that is independent of IMWG parameters alone. This information is potentially clinically actionable and further studies are needed to investigate if switching treatment regimens early based on cfDNA translates into better PFS, overall survival and less complications for patients.

We propose that cfDNA interrogation should be incorporated in clinical trials to further establish its value as an independent predictive marker across therapeutic regimens.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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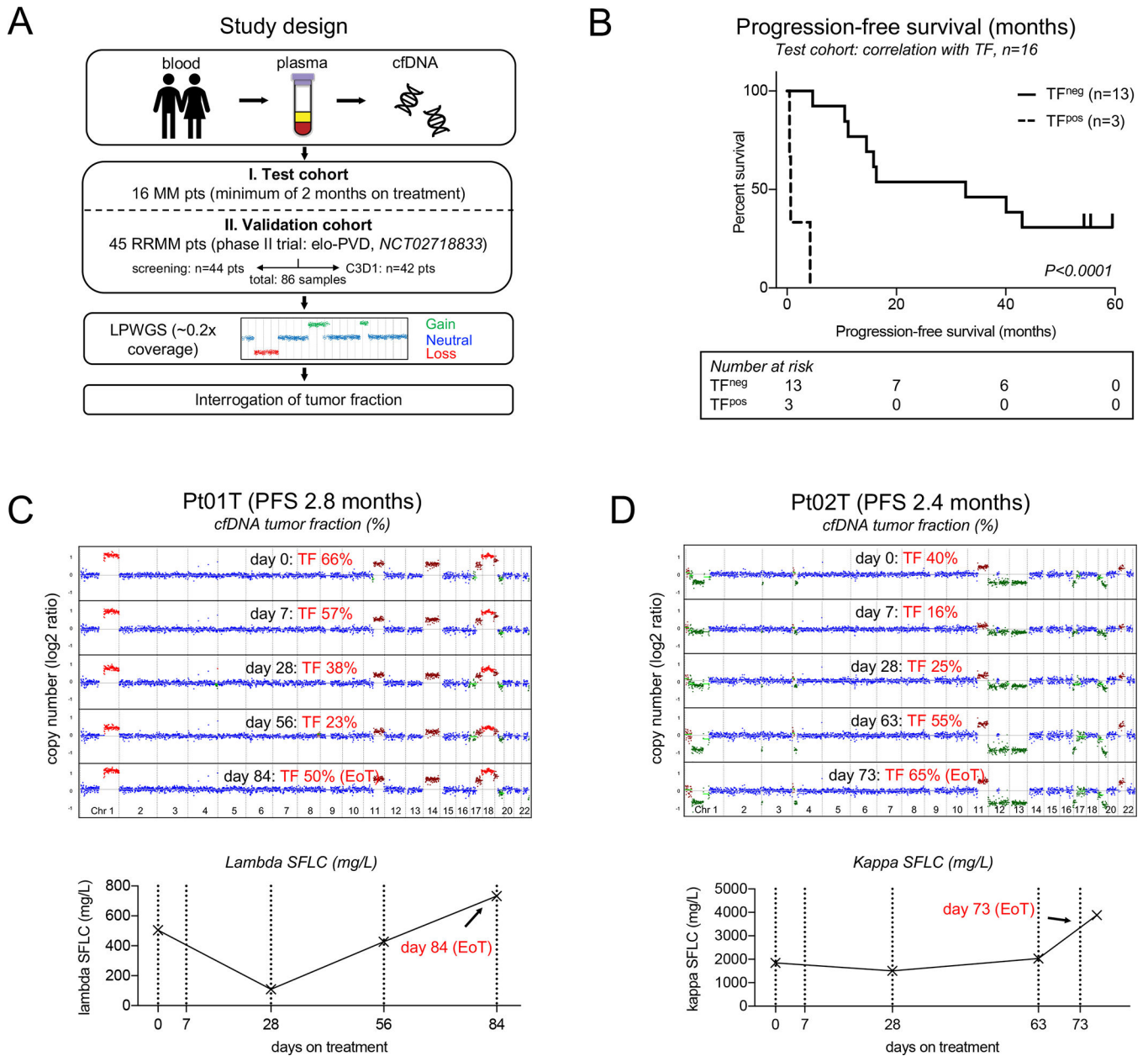
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**Figure 1. Tumor fraction in cfDNA as proxy for tumor burden and as prognostic marker.**

**A.** Primary analysis of cfDNA in a test cohort of MM patients (n=16) undergoing a variety of therapeutic regimens. Tumor fraction in cfDNA was measured after a minimum of two months on the current regimen using *ichorCNA*. In a second validation cohort, patients with RRMM (n=45) received uniform treatment with elotuzumab, pomalidomide, bortezomib and dexamethasone (elo-PVD, [clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02718833) identifier: NCT02718833). Tumor fraction in cfDNA was measured at baseline (n=44 available), and at cycle 3 day 1 (C3D1, n=42 available) of treatment. **B.** Kaplan-Meier curve for PFS (months) in MM patients with tumor fraction positivity (>10%) or negativity (<10%) in cfDNA. **C,D.** Serial copy number profile, cfDNA tumor fraction and iFLC obtained at multiple time points in two patients, Pt01T (**C**, PFS 2.8 months) and Pt02T (**D**, PFS, 2.4 months). Abbreviations: RRMM= relapsed/

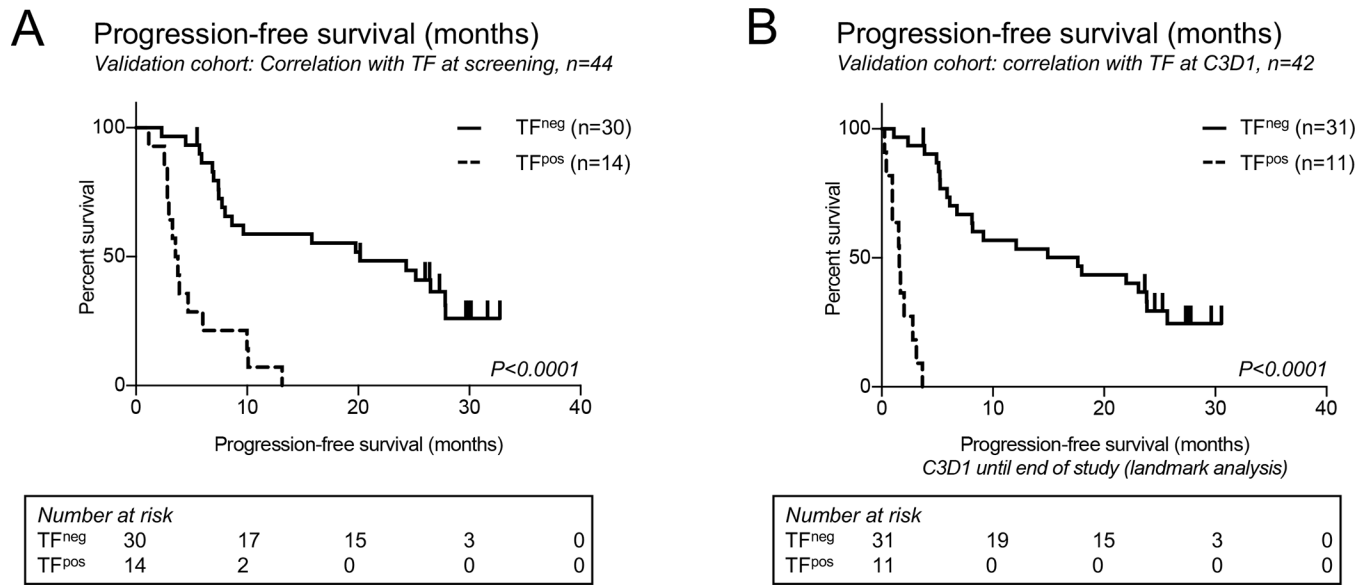
refractory multiple myeloma, LPWGS= low-pass whole genome sequencing, elo-PVD= elotuzumab-pomalidomide-bortezomib-dexamethasone, C3D1= cycle 3 day 1 of elo-PVD treatment, TF= tumor fraction, EoT= end of treatment.

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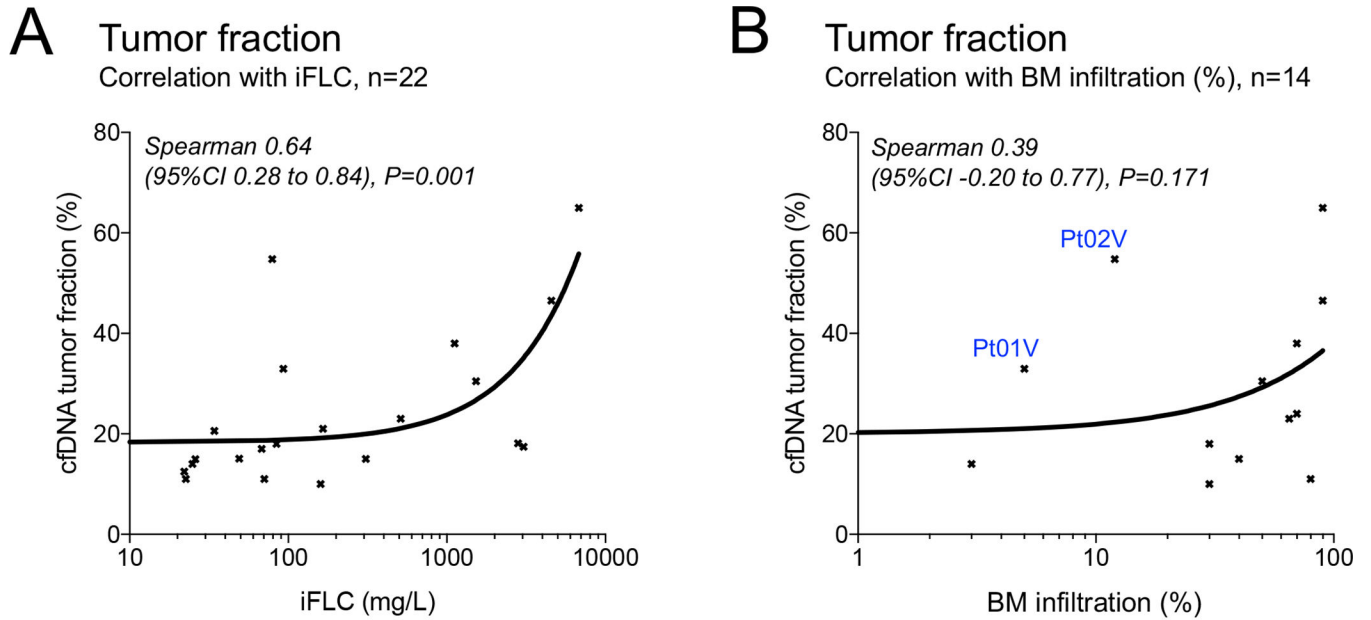
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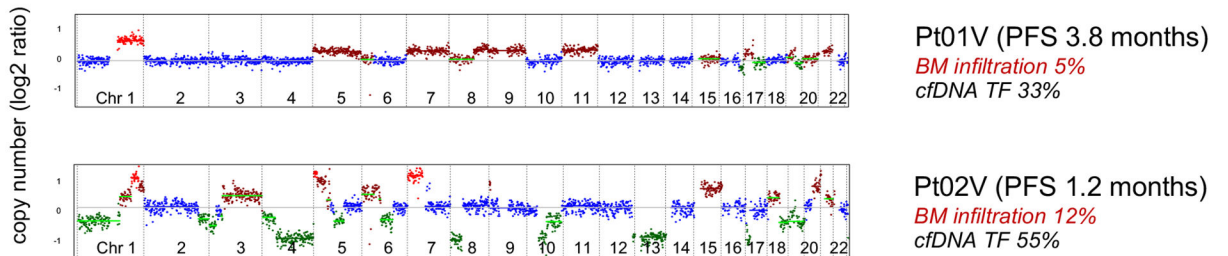


**Figure 2. Tumor fraction in cfDNA is predictive of progression-free survival.**

Kaplan-Meier survival for PFS (months) in MM patients with tumor fraction positive or negative results at screening (**A**, n=44) and C3D1 (**B**, n=42). Abbreviations: TF= tumor fraction.

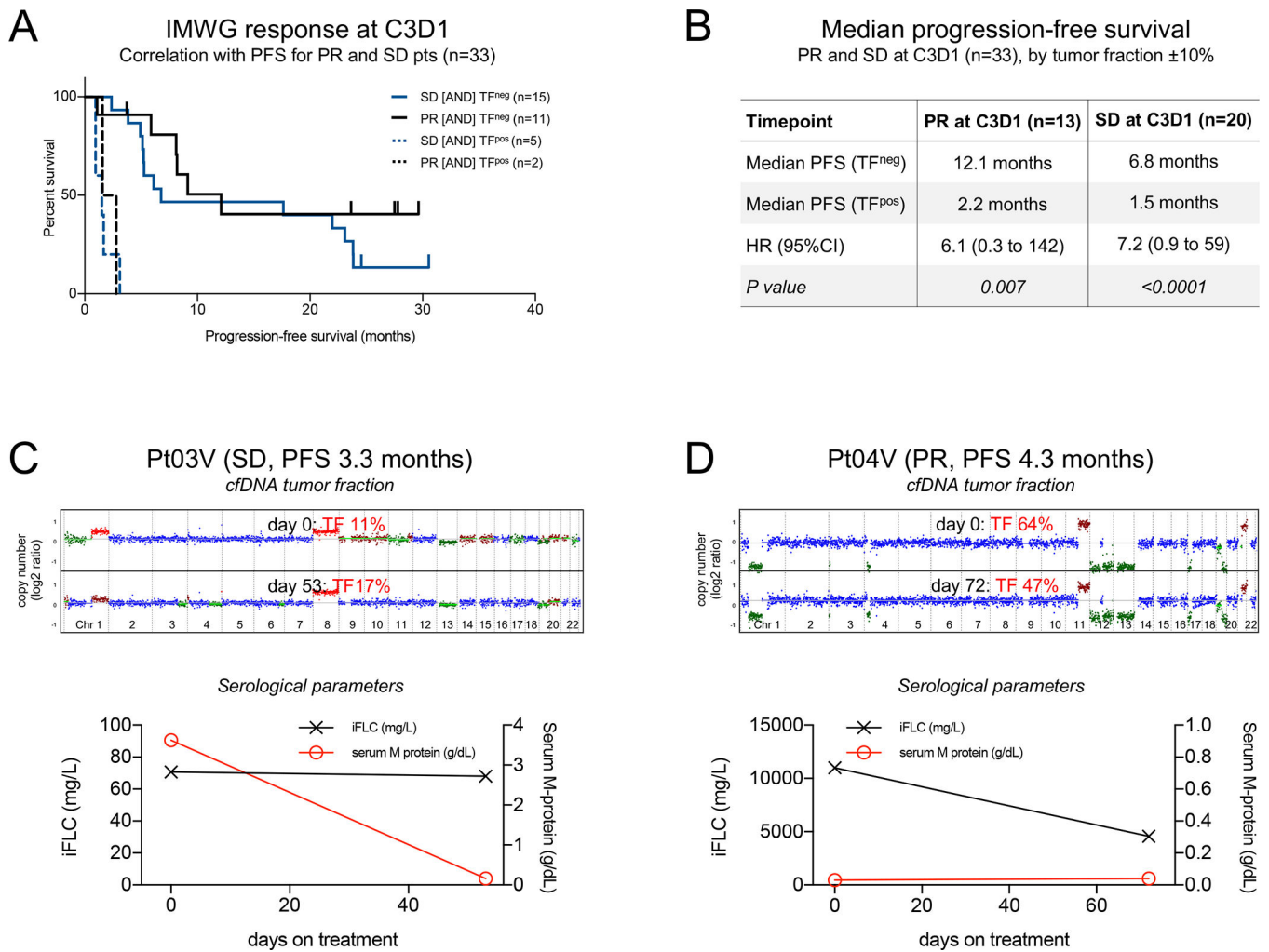


**C** Patients with diverging BM and cfDNA results  
BM biopsy-paired copy number plots from cfDNA at screening and respective outcomes



**Figure 3. Comparing MM-derived cfDNA with serological parameters and bone marrow infiltration.**

Correlation of paired samples between cfDNA tumor fraction and iFLC (**A**, n=79) or bone marrow infiltration (**B**, n=56). **C**. Copy number profile and matched bone marrow result for Pt01V (PFS 3.8 months) and Pt02V (PFS 1.2 months). Abbreviations: iFLC= involved serum-free light chain, BM= bone marrow, PFS= progression-free survival, TF= tumor fraction.

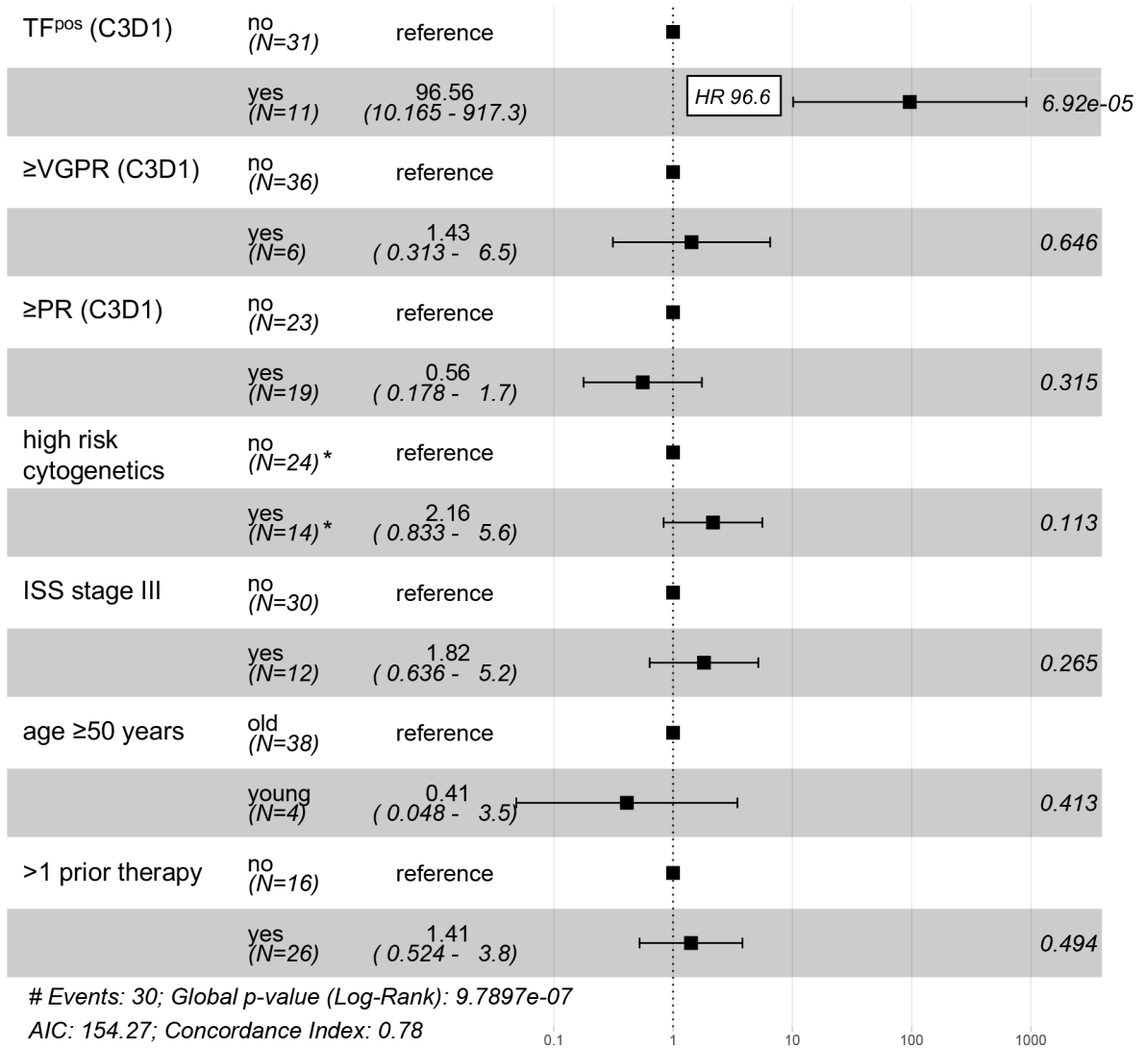


**Figure 4. Refining IMWG response criteria with cfDNA as an orthogonal marker of response.**

**A.** Kaplan-Meier survival for PFS (months) in 33 MM pts with SD or PR according to IMWG criteria separated by tumor fraction positive or negative cfDNA testing at C3D1.

**B.** Detailed median PFS and hazard ratio values for patients with SD or PR according to respective cfDNA status at C3D1. **C,D.** Copy number profile, cfDNA tumor fraction, iFLC and serum M-protein over time in two patients, Pt03V (**C**, PFS 3.3 months) and Pt04V (**D**, PFS 4.3 months). **Abbreviations:** IMWG= International Myeloma Working Group, C3D1= cycle 3 day 1 (of elo-PVD treatment), PFS= progression-free survival, PR= partial response, SD= stable disease, TF= tumor fraction.

### Progression-free survival (months) Multivariate hazard model for cfDNA tumor fraction at C3D1, n=42



**Figure 5. Cox proportional hazard model for PFS and cfDNA tumor fraction after two cycles of elo-PVD.**  
Forest plots of hazard ratios for PFS according to cfDNA positivity (P=6.92e-05) at cycle 3 day 1 (C3D1) compared to achievement of VGPR (P=0.646) and PR (P=0.315) at the same time point (n=42), as well as age, having received >1 treatment, ISS stage III and high-risk cytogenetics. Cytogenetic information was not available for n=4 patients (\*). Abbreviations: C3D1= cycle 3 day 1 (of elo-PVD treatment), TF= tumor fraction, VGPR= very good partial response, PR= partial response, SD= stable disease, ISS= International Staging System, HR= high-risk (del17p, t(4;14), t(14;16), t(14;20)).



**Table 1.**

Patient characteristics (test cohort, n=16)

<i>Tumor fraction threshold</i>	<b>TF<sup>pos</sup></b>	<b>TF<sup>neg</sup></b>
<i>Size</i>	n=3	n=13
<i>Current treatment</i>		
Trametinib	2	
Trametinib-dabrafenib	1	
RVD		6
VCd		3
KRD		1
Isa-Pom-Dex		1
Ixa-Dex		1
Rico-Pom-Dex		1
<i>PFS from time of measurement (median)</i>	<u>0.7 (0.5–4.2) months</u>	<u>32.7 (4.7–55.5) months</u>
HR (95%CI)	9.9 (0.4 to 238)	
P value	<0.0001	

**Abbreviations:** TF= tumor fraction, RVD= lenalidomide-bortezomib-dexamethasone, VCd= bortezomib-cyclophosphamide-dexamethasone, KRD= carfilzomib-lenalidomide-dexamethasone, Isa-Pom-Dex= isatuximab-pomalidomide-dexamethasone, Ixa-Dex= ixazomib-dexamethasone, Rico-Pom-Dex= ricolinostat-pomalidomide-dexamethasone, HR= hazard ratio

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**Table 2.**

Patient characteristics by cfDNA after two months of treatment (validation cohort, n=42)

<i>Tumor fraction threshold</i>	<b>C3D1 TF<sup>pos</sup></b>	<b>C3D1 TF<sup>neg</sup></b>	<b>P value</b>
<i>Size</i> *	n=11	n=31	
<i>Sex</i>			
Female	1 (9%)	10 (32%)	0.139
Male	10 (91%)	21 (68%)	
<i>Age, years</i> (median range)	70 (60–80)	64 (40–79)	0.033
<i>ISS stage</i>			
I	1 (9%)	11 (35%)	
II	2 (18%)	11 (35%)	0.243 (ISS III vs. not)
III	5 (45%)	7 (23%)	
unknown	3 (27%)	2 (6%)	
<i>Cytogenetics unfavorable</i>	2 (18%)	12 (39%)	
del17p	2 (18%)	7 (23%)	
t(4;14)	0	2 (6%)	0.283 (unfavorable vs. not)
t(14;16)	1 (9%)	1 (3%)	
t(14;20)	0	2 (6%)	
standard risk	7 (64%)	17 (55%)	
unknown	2 (18%)	2 (6%)	
<i>EM-MM (n, %)</i>	2 (18%)	1 (3%)	0.163
<i>Prior lines of therapy</i> (median, range)	4 (1–8)	2 (1–10)	0.294
<i>PFS</i> ** on trial (median, range)	1.6 months	17.6 months	
HR (95%CI)	7.6 (1.8 to 31.3)		<0.0001
<i>BM infiltration</i> ***, % (median, range)	80% (70–90%) *	20% (1–90%)	0.024
<i>iFLC level, mg/L</i> (median, range)	594 (22–5012)	46 (1–1670)	0.001
<i>Serum M protein g/dL</i> (median, range)	0.5 (0–2.2)	0.5 (0–10)	0.525
<i>Serum LDH, U/L</i> (median, range)	184 (146–274)	153 (119–400)	0.457

**Abbreviations:** TF= tumor fraction, ISS= International Staging System, EM-MM= extramedullary multiple myeloma, PFS= progression-free survival, HR= hazard ratio, BM= bone marrow, iFLC= involved serum-free light chain

\* cfDNA results from C3D1 available for n=42 patients

\*\* PFS calculated as time from last measurement (C3D1) to progression (landmark analysis)

\*\*\* BM results from C3D1 available for n=18 patients