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# High-dose carfilzomib achieves superior anti-tumor activity over low-dose and recaptures response in relapsed/refractory multiple myeloma resistant to low-dose carfilzomib by co-inhibiting the $\beta$ 2 and $\beta$ 1 subunits of the proteasome complex

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## Abstract

Optimal carfilzomib dosing is a matter of debate. We analyzed the inhibition profiles of proteolytic proteasome subunits  $\beta$ 5,  $\beta$ 2 and  $\beta$ 1 after low-dose (20/27 mg/m<sup>2</sup>) versus high-dose ( $\geq$ 36 mg/m<sup>2</sup>) carfilzomib in 103 pairs of peripheral blood mononuclear cells from patients with relapsed/refractory (RR) multiple myeloma (MM).  $\beta$ 5 activity was inhibited (median inhibition  $>$ 50%) *in vivo* by 20 mg/m<sup>2</sup>, whereas  $\beta$ 2 and  $\beta$ 1 were co-inhibited only by 36 and 56 mg/m<sup>2</sup>, respectively. Co-inhibition of  $\beta$ 2 ( $P=0.0001$ ) and  $\beta$ 1 activity ( $P=0.0005$ ) differed significantly between high-dose and low-dose carfilzomib. Subsequently, high-dose carfilzomib showed significantly more effective proteasome inhibition than low-dose carfilzomib *in vivo* ( $P=0.0003$ ). We investigated the clinical data of 114 patients treated with carfilzomib combinations. High-dose carfilzomib demonstrated a higher overall response rate ( $P=0.03$ ) and longer progression-free survival (PFS) ( $P=0.007$ ) than low-dose carfilzomib. Therefore, we escalated the carfilzomib dose to  $\geq$ 36 mg/m<sup>2</sup> in 16 patients who progressed during low-dose carfilzomib-containing therapies. High-dose carfilzomib recaptured response ( $\geq$  partial remission) in nine (56%) patients with a median PFS of 4.4 months. Altogether, we provide the first *in vivo* evidence in RRMM patients that the molecular activity of high-dose carfilzomib differs from that of low-dose carfilzomib by co-inhibition of  $\beta$ 2 and  $\beta$ 1 proteasome subunits and, consequently, high-dose carfilzomib achieves a superior anti-MM effect than low-dose carfilzomib and recaptures the response in RRMM resistant to low-dose carfilzomib. The optimal carfilzomib dose should be  $\geq$ 36 mg/m<sup>2</sup> to reach a sufficient anti-tumor activity, while the balance between efficacy and tolerability should be considered in each patient.

## Introduction

The proteasome is a multi-subunit complex that is responsible for intracellular protein degradation. Only three proteasome subunits harbor proteolytic activity,  $\beta$ 1 (caspase-like),  $\beta$ 2 (trypsin-like), and  $\beta$ 5 (chymotrypsin-like), which cleave peptide bonds C-terminally of acidic, basic,

and hydrophobic amino acid residues, respectively.<sup>1</sup> Currently, proteasome inhibition is a major treatment strategy for multiple myeloma (MM).<sup>2</sup> All currently approved proteasome inhibitors (PI) primarily target the rate-limiting  $\beta$ 5 subunit.<sup>3</sup> Carfilzomib (CFZ), a second-generation epoxyketone-based PI selectively targets the  $\beta$ 5 subunit at low concentrations but co-inhibits the  $\beta$ 2 and  $\beta$ 1 subunits

only at high concentrations, which subsequently enhances the cytotoxic activity against MM *in vitro*.<sup>4</sup>

CFZ-containing therapies have shown outstanding anti-MM activity in patients with relapsed/refractory (RR) MM.<sup>5-10</sup> As of August 2022, CFZ has been approved for RRMM in various combination regimens, such as Kd (CFZ and dexamethasone), KRd (CFZ, lenalidomide, and dexamethasone), and D-Kd (daratumumab, CFZ, and dexamethasone).<sup>11</sup> However, with the approved dose ranging from low-dose (20-27 mg/m<sup>2</sup>) to high-dose (up to 70 mg/m<sup>2</sup> once weekly or twice weekly), optimal CFZ dose is still a matter of debate. In clinical practice, the relationship between CFZ dose and inhibition profiles of proteasome subunits is largely unknown. Moreover, it remains to be explored whether CFZ dose escalation may recapture the clinical response in RRMM patients progressing under low-dose CFZ-containing treatments. Furthermore, real-world data on high-dose CFZ are still very limited for the currently approved Kd, KRd, and D-Kd combination regimens. Therefore, the aim of the current study was to address these issues by analyzing the inhibition profiles of proteolytic proteasome subunits  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5 in RRMM treated with different CFZ doses and to investigate the clinical efficacy and safety of high-dose CFZ in RRMM treated with Kd, KRd, and D-Kd combinations. In addition, we aimed to evaluate CFZ dose escalation treatment in patients with RRMM resistant to low-dose CFZ-containing treatments.

## Methods

### Patients

First, 103 pairs of peripheral blood mononuclear cell (PBMC) samples were collected before and 3 hours after CFZ treatment from RRMM patients (defined by the current International Myeloma Working Group guidelines<sup>12</sup>) and were included in the study. The patient cohort characteristics are presented in Table 1. Second, the clinical data of 114 RRMM patients treated with CFZ combination regimens (Kd, KRd, and D-Kd) were investigated. The patient characteristics are summarized in Table 2. Third, the clinical data of 16 heavily pretreated RRMM patients, in whom we escalated CFZ dosing due to progression during low-dose CFZ-containing therapy, were analyzed. The characteristics of these patients and their CFZ-containing treatment regimens are shown in Table 3 and the *Online Supplementary Table S1*. Patient demographics, MM-related data, therapy responses, adverse events (AE), and survival outcomes were investigated. AE during treatment were classified according to the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0. All procedures were performed in accordance with the Declaration of Helsinki and the national ethical standards. Informed consent was obtained from all patients included in the study.

### Sampling and sample preparation

Proteasome inhibition in PBMC largely mirrors proteasome inhibition in plasma cells *in vivo*.<sup>4,13</sup> Therefore, we analyzed proteasome inhibition in PBMC of RRMM patients in our study. The detailed description of sampling and sample preparation is provided in the *Online Supplementary Appendix*.

**Table 1.** Characteristics of 103 relapsed/refractory multiple myeloma patients treated with respective carfilzomib doses.

Parameter	
Patients, N	103
Sex, N (%)	
Male	55 (53.4)
Female	48 (46.6)
Age in years at sampling (range)	64 (41-83)
Subtype, N (%)	
IgG	61 (59.2)
Non-IgG	30 (29.1)
LC	12 (11.7)
EMD, N (%)	47 (45.6)
EMD adjacent to bone	13 (12.6)
EMD without bone contact	34 (33.0)
Cytogenetics, N (%)	
High risk <sup>#</sup>	59 (57.3)
Standard-risk	44 (42.7)
Prior lines of therapies at sampling, N(%)	
1-3	50 (48.6)
4-6	30 (29.1)
>6	23 (22.3)
Drug exposure at sampling, N (%)	
IMiDs	
Lenalidomide	100 (97.1)
Pomalidomide	72 (69.9)
PI	
Bortezomib	89 (86.4)
Carfilzomib	97 (94.2)
Monoclonal antibody	
Elotuzumab	37 (35.9)
Daratumumab	87 (84.5)
Prior SCT	
Autologous SCT	85 (82.5)
Allogeneic SCT	14 (13.6)
BCMA-directed therapy	
ADC	3 (2.9)
CAR T cell	5 (4.9)
Bispecific antibody	2 (1.9)
CFZ dosing, N (%)	
20 mg/m <sup>2</sup>	23 (22.3)
27 mg/m <sup>2</sup>	27 (26.2)
36 mg/m <sup>2</sup>	38 (36.9)
56 mg/m <sup>2</sup>	15 (14.6)

ADC: antibody drug conjugate; BCMA: B-cell maturation antigen; CAR T cell: chimeric antigen receptor modified T cell; CFZ: carfilzomib; EMD: extramedullary disease; ImiD: immunomodulatory drug; LC: light chain; PI: proteasome inhibitor; RRMM: relapsed/refractory multiple myeloma; SCT: stem cell transplant; <sup>#</sup>defined as the presence of at least one of the following: t(4;14), t(14;16), t(14;20), del17p and amp1q21.

**Table 2.** Characteristics of 114 relapsed/refractory multiple myeloma patients treated with Kd, KRd or D-Kd.

Basic characteristics					
Parameter	All	Kd	KRD	D-Kd	P
Patients, N	114	33	71	10	
Sex, N (%)					0.31
Male	76 (66.7)	17 (51.5)	51 (71.8)	8 (80.0)	
Female	38 (33.3)	16 (48.5)	20 (28.2)	2 (20.0)	
Age in years at diagnosis of MM, median (range)	58 (34-81)	58 (45-73)	57 (34-81)	56 (44-71)	0.69
Age in years at Kd/KRD/D-Kd, median (range)	63 (39-83)	65 (54-76)	62 (39-83)	63 (52-80)	0.33
Subtype, N (%)					0.12
IgG	68 (59.6)	22 (66.7)	41 (57.8)	5 (50.0)	
non-IgG	32 (28.1)	8 (24.2)	19 (26.8)	5 (50.0)	
LC	14 (12.3)	3 (9.1)	11 (15.5)	0 (0.0)	
Cytogenetics, N (%)					0.21
High-risk <sup>#</sup>	63 (55.2)	18 (54.5)	28 (39.4)	7 (70.0)	
Standard-risk	51 (44.8)	15 (45.5)	43 (60.6)	3 (30.0)	
Prior lines of therapy, N (%)					0.22
1-3	72 (63.1)	17 (51.5)	48 (67.6)	7 (70.0)	
4-6	28 (24.6)	9 (27.3)	17 (23.9)	2 (20.0)	
>6	14 (12.3)	7 (21.2)	6 (8.5)	1 (10.0)	
Pretreatment prior to the current line of therapy, N (%)	Exposed/Refractory	Exposed/Refractory	Exposed/Refractory	Exposed/Refractory	
IMiDs	80 (70.2)/50 (43.9)	28 (84.8)/19 (57.6)	43 (60.6)/26 (36.6)	9 (90.0)/5 (50.0)	0.15/0.25
Lenalidomide	80 (70.2)/40 (35.1)	28 (84.8)/13 (39.4)	43 (60.6)/23 (32.4)	9 (90.0)/4 (40.0)	0.15/0.24
Pomalidomide	38 (33.3)/34 (29.9)	16 (48.5)/14 (42.4)	17 (23.9)/15 (21.1)	5 (50.0)/5 (50.0)	0.38/0.39
PI	106 (92.9)/53 (46.5)	28 (84.8)/17 (51.5)	68 (95.8)/32 (45.1)	10 (100.0)/4 (40.0)	0.48/0.08
Bortezomib	104 (91.2)/49 (42.9)	27 (81.8)/16 (48.5)	67 (94.4)/30 (42.3)	10 (100.0)/3 (30.0)	0.47/0.11
Carfilzomib	18 (15.8)/12 (10.5)	6 (18.2)/3 (9.1)	8 (11.3)/5 (7.0)	4 (40.0)/4 (40.0)	0.46/0.49
Monoclonal antibodies					
Daratumumab	32 (28.1)/30 (26.3)	9 (27.3)/9 (27.3)	17 (23.9)/16 (22.5)	6 (60.0)/5 (60.0)	0.36/0.36
Elotuzumab	18 (15.8)/12 (10.5)	7 (21.2)/4 (12.1)	10 (14.1)/7 (9.9)	1 (10.0)/1 (10.0)	0.40/0.43
Prior SCT, N(%)					
Autologous SCT	100 (87.7)	29 (87.9)	62 (87.3)	9 (90.0)	0.40
Allogeneic SCT	6 (5.3)	0 (0.0)	6 (8.5)	0 (0.0)	0.43
Treatment response and toxicity					
Parameter	All	Kd	KRD	D-Kd	P
Number of cycles, median (range)		3 (1-10)	3 (1-20)	3 (1-5)	0.11
Maximum CFZ dose, N (%)					0.35
15 mg/m <sup>2</sup>	5 (4.4)	2 (6.1)	3 (4.2)	0 (0.0)	
20 mg/m <sup>2</sup>	8 (7.0)	4 (12.1)	3 (4.2)	1 (10.0)	
27 mg/m <sup>2</sup>	64 (56.1)	7 (21.2)	54 (76.1)	3 (30.0)	
36 mg/m <sup>2</sup>	9 (7.9)	1 (3.0)	7 (9.9)	1 (10.0)	
45 mg/m <sup>2</sup>	1 (0.9)	0 (0.0)	1 (1.4)	0 (0.0)	
56 mg/m <sup>2</sup>	27 (23.7)	19 (57.6)	3 (4.2)	5 (50.0)	
Maximum LEN dose (QD), N (%)					
5 mg		/	8 (11.3)	/	
10 mg		/	10 (14.1)	/	
15 mg		/	13 (18.3)	/	
20 mg		/	2 (2.8)	/	
25 mg		/	38 (53.5)	/	
Best response, N (%)					0.04
CR	7 (6.1)	1 (3.0)	4 (5.6)	2 (20.0)	
VGPR	29 (25.4)	4 (12.1)	24 (33.8)	1 (10.0)	
PR	36 (31.6)	11 (33.3)	22 (31.0)	3 (30.0)	
MR	19 (16.7)	8 (24.3)	10 (14.1)	1 (10.0)	
PD	21 (18.4)	8 (24.3)	10 (14.1)	3 (30.0)	
na	2 (1.8)	1 (3.0)	1 (1.4)	0 (0.0)	

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Treatment response and toxicity					
Parameter	All	Kd	KRD	D-Kd	P
Non-hematologic toxicities grade $\geq 3$ , N (%)					
Cardiotoxicity	11 (9.6)	5 (15.2)	6 (8.5)	/	
Pneumonia	5 (4.4)	/	5 (7.0)	/	
Fever of unknown origin	3 (2.6)	3 (9.1)	/	/	
Respiratory infection	11 (9.6)	5 (15.1)	6 (8.5)	/	
Fatigue	2 (1.8)	2 (6.1)	/	/	
Renal failure	4 (3.5)	1 (3.0)	3 (4.2)	/	
Thromboembolic events	1 (0.9)	1 (3.0)	/	/	
Catheter associated infection	1 (0.9)	1 (3.0)	/	/	
Liver enzyme elevated	2 (1.8)	/	2 (2.8)	/	
Bacterial meningitis	1 (0.9)	/	1 (1.4)	/	
Delirium	1 (0.9)	/	1 (1.4)	/	
Infection of urinary tract	2 (1.8)	/	1 (1.4)	1 (10.0)	
Herpes zoster	1 (0.9)	/	1 (1.4)	/	
Soft tissue infection	1 (0.9)	/	/	1 (10.0)	
Hematologic toxicities grade $\geq 3$ , N (%)					
Anemia	29 (25.4)	6 (18.2)	19 (26.8)	4 (40.0)	0.29
Leukopenia	34 (29.8)	7 (21.2)	21 (29.6)	6 (60.0)	0.30
Thrombocytopenia	37 (32.4)	14 (42.4)	18 (25.4)	5 (50.0)	0.35
Neutropenia	30 (26.3)	2 (6.1)	23 (32.4)	5 (50.0)	0.30

CFZ: carfilzomib; CR: complete remission; D-Kd: daratumumab, carfilzomib, dexamethasone; ImiDs: immunomodulatory drugs; Kd: carfilzomib, dexamethasone; KRD: carfilzomib, lenalidomide, dexamethasone; LC: light chain; LEN: lenalidomide; MM: multiple myeloma; MR: minor response; na: not available; PD: progressive disease; PI: proteasome inhibitors; PR: partial remission; SCT: stem cell transplant; VGPR: very good partial remission; #defined as presence of at least one of the following: del(17p), amp1q21, t(4;14), t(14;16) and t(14;20). IgG: immunoglobulin G.

### Proteasome $\beta$ -subunits profiling with activity-based proteasome probes labeling

Proteasome subunit activity was assessed using protein lysate from PBMC. Briefly, PBMC pellets were lysed with a lysis buffer. The lysates were then labeled for 1 hour at 37°C with subunit-selective, fluorescent, activity-based proteasome probes (ABPP) that visualized  $\beta 1$ ,  $\beta 2$ , and  $\beta 5$  activity of the constitutive proteasome and immune proteasomes as previously described by de Bruin *et al.*<sup>14</sup> (*Online Supplementary Figure S1*), and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In order to limit variability, samples from respective patients were always run on the same gel and to minimize differences in gel exposure, a sample prepared from a pool of PBMC obtained from healthy donors was run on each gel. After SDS-PAGE, gel images were acquired using Fusion Solo S Western Blot and Chemi Imaging System (Vilber Lourmat, Collégien, France). Active proteasome subunits were quantitatively assessed in each sample by densitometry using Fiji (an open-source image processing package based on ImageJ)<sup>15</sup> and normalized to a fluorescence intensity obtained from the PBMC sample on each gel. For each sample, the activity of each proteolytically active  $\beta$  subunit was calculated by summarizing the normalized band fluorescence intensity of the respective constitutive (c) proteasome subunit and the corresponding subunit of the immuno (i) proteasomes (i.e.,  $\beta 1c+i$ ,  $\beta 2c+i$ , and  $\beta 5c+i$ ). The

inhibition of subunit activity after CFZ exposure in relation to a paired sample before CFZ exposure was calculated for each individual patient (Figure 1A, B). Total proteasome activity was defined as the average activity of the  $\beta 1c+i$ ,  $\beta 2c+i$ , and  $\beta 5c+i$  proteasome subunits. For further analysis, we dichotomized the patients into two groups: low-dose ( $\leq 27$  mg/m<sup>2</sup>) and high-dose ( $\geq 36$  mg/m<sup>2</sup>) CFZ.

### Kd, KRD, and D-Kd regimens

A detailed description of the drug administrations and treatment regimens is provided in the *Online Supplementary Appendix*.

### Statistical evaluation

Statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at 0.05 ( $P$  value  $< 0.05$ ). A more specific description of the statistical evaluation used in this study is provided in *Online Supplementary Appendix*.

## Results

### Inhibition profile of proteolytic proteasome subunits at different carfilzomib doses in relapsed/refractory multiple myeloma patients

CFZ has a very short half-life of  $< 30$  minutes (min) and

reaches full proteasome inhibition within 60-90 min after CFZ treatment in peripheral tissues *in vivo*.<sup>16</sup> Additionally, *in vitro*, proteasome activity recovers to baseline after 24 hours from CFZ treatment and remains largely constant at later time points.<sup>4</sup> Therefore, we investigated proteolytic proteasome subunit activity in PBMC before and 3 hours after CFZ treatment, and we combined the activity of constitutive (c) and immuno-proteasome (i), i.e.,  $\beta 1c+i$ ,  $\beta 2c+i$ , and  $\beta 5c+i$ . In our cohort, 23, 27, 38, and 15 patients received 20, 27, 36, and 56 mg/m<sup>2</sup> of CFZ, respectively (Table 1). All 103 patients were treated with twice-weekly CFZ regimen, and the PBMC samples were collected on day 1 before and 3 hours after CFZ infusion. Generally, proteasome subunit activity decreased with increasing CFZ dose, with the strongest inhibition of  $\beta 5c+i$ , followed by  $\beta 2c+i$ , and  $\beta 1c+i$  at all dose levels (Figure 1B). Biologically meaningful inhibition (median residual activity <50% of baseline) of  $\beta 5c+i$  was already achieved with 20 mg/m<sup>2</sup>, whereas  $\beta 2c+i$  and  $\beta 1c+i$  activity remained largely unchanged at this dosing level. Meaningful co-inhibition of  $\beta 2c+i$  and  $\beta 1c+i$  was observed only at higher doses of 36 and 56 mg/m<sup>2</sup>, respectively. Interestingly, the active  $\beta 2c+i$  and  $\beta 1c+i$  subunits were moderately upregulated upon  $\beta 5c+i$  inhibition by CFZ only at 20 mg/m<sup>2</sup>, possibly contributing to compensatory activity. In patients treated with 56 mg/m<sup>2</sup>, all  $\beta 1c+i$ ,  $\beta 2c+i$ , and  $\beta 5c+i$  were inhibited by >50% compared to baseline before CFZ. We noticed a significant difference in  $\beta 2c+i$  and  $\beta 1c+i$  subunit inhibition between the groups treated with 20 mg/m<sup>2</sup> versus 36 mg/m<sup>2</sup> with a median residual activity of  $\beta 2c+i$  of 120.1% (95% confidence interval [CI]: 76.8-174.6) versus 47.2% (95% CI: 31.2-53.8;  $P < 0.0001$ ); median residual activity of  $\beta 1c+i$  of 132.8% (95% CI: 62.4-188.0) versus 60.7% (95% CI: 39.3-77.1;  $P = 0.0009$ ). The same held true for the groups CFZ treated with 27 mg/m<sup>2</sup> versus 56 mg/m<sup>2</sup> with a median residual activity of  $\beta 2c+i$  of 65.9% (95% CI: 32.5-88.4) versus 39.5% (95% CI: 17.8-54.9;  $P = 0.035$ ); median residual activity of  $\beta 1c+i$  of 81.2% (95% CI: 53.8-116.9) versus 42.1% (95% CI: 23.8-69.5;  $P = 0.017$ ). In contrast,  $\beta 5c+i$  inhibition did not differ significantly between the groups at 20 mg/m<sup>2</sup> versus 36 mg/m<sup>2</sup> or 27 mg/m<sup>2</sup> versus 56 mg/m<sup>2</sup> ( $P > 0.05$ ). In terms of total proteasome inhibition (average of  $\beta 1c+i$ ,  $\beta 2c+i$ , and  $\beta 5c+i$ ), we observed a significant difference between the groups treated with 20 mg/m<sup>2</sup> versus 36 mg/m<sup>2</sup> with a median residual total proteasome activity of 91.7% (95% CI: 56.1-133.1) versus 41.7% (95% CI: 28.2-52.7;  $P = 0.0003$ ). Similarly, CFZ treatment with 56 mg/m<sup>2</sup> showed significantly superior total proteasome inhibition over 27 mg/m<sup>2</sup> with a median residual total proteasome activity of 55.0% (95% CI: 37.8-86.7) versus 33.2% (95% CI: 23.8-35.7;  $P = 0.019$ ). We then dichotomized the CFZ dosing into two groups: low-dose ( $\leq 27$  mg/m<sup>2</sup>) and high-dose ( $\geq 36$  mg/m<sup>2</sup>) CFZ. Between both groups,  $\beta 2c+i$  and  $\beta 1c+i$  inhibition significantly differed

**Table 3.** Characteristics of patients with relapsed/refractory multiple myeloma progressing from low-dose carfilzomib and treated with carfilzomib dose escalation.

Parameter	
Patients, N	16
Sex, N (%)	
Male	10 (62)
Female	6 (38)
Age in years at diagnosis of MM, median (range)	65 (41-79)
Age in years at CFZ dose escalation, median (range)	71 (45-83)
Time in months between diagnosis of MM and CFZ dose escalation, median (range)	61 (22-144)
Subtype, N (%)	
IgG	11 (69)
non-IgG	5 (31)
Cytogenetics, N (%)	
High-risk <sup>#</sup>	11 (69)
Standard-risk	5 (31)
EMD, N (%)	
EMD without bone contact	6 (38)
no EMD	10 (62)
Prior lines of therapy, N (%)	
2-4	5 (31)
5-7	5 (31)
$\geq 8$	6 (38)
Pretreatment prior to the current line of therapy, N (%)	Exposed/Refractory
IMiDs	15 (94)/13 (81)
Lenalidomide	15 (94)/11 (69)
Pomalidomide	14 (88)/13 (81)
PI	16 (100)/15 (94)
Bortezomib	16 (100)/14 (88)
Carfilzomib (low-dose)	8 (50)/8 (50)
Monoclonal antibodies	
Daratumumab	15 (94)/15 (94)
Elotuzumab	3 (19)/3 (19)
Penta-refractory <sup>†</sup>	6 (38)
Prior SCT	
Autologous SCT	12 (75)
BCMA-directed therapy	
ADC	2 (13)
CAR T cell	2 (13)
BITE	1 (6)
CFZ dose escalation, N(%)	
15→36 mg/m <sup>2</sup>	1 (6)
20→56 mg/m <sup>2</sup>	1 (6)
27→36 mg/m <sup>2</sup>	6 (38)
27→56 mg/m <sup>2</sup>	8 (50)
Best response to CFZ dose escalation, N (%)	
VGPR	5 (31)
PR	4 (25)
MR	6 (38)
PD	1 (6)
Non-hematologic toxicities grade $\geq 3$ after CFZ dose escalation, N(%)	
Pneumonia	4 (25)
Fever of unknown origin	2 (13)
Fatigue	1 (6)
Cardiotoxicity	1 (6)
Corona virus infection HKU1	1 (6)
Renal failure	1 (6)
Hematologic toxicities grade $\geq 3$ after CFZ dose escalation, N(%)	
Anemia	10 (63)
Leukopenia	10 (63)
Thrombocytopenia	8 (50)
Neutropenia	4 (25)

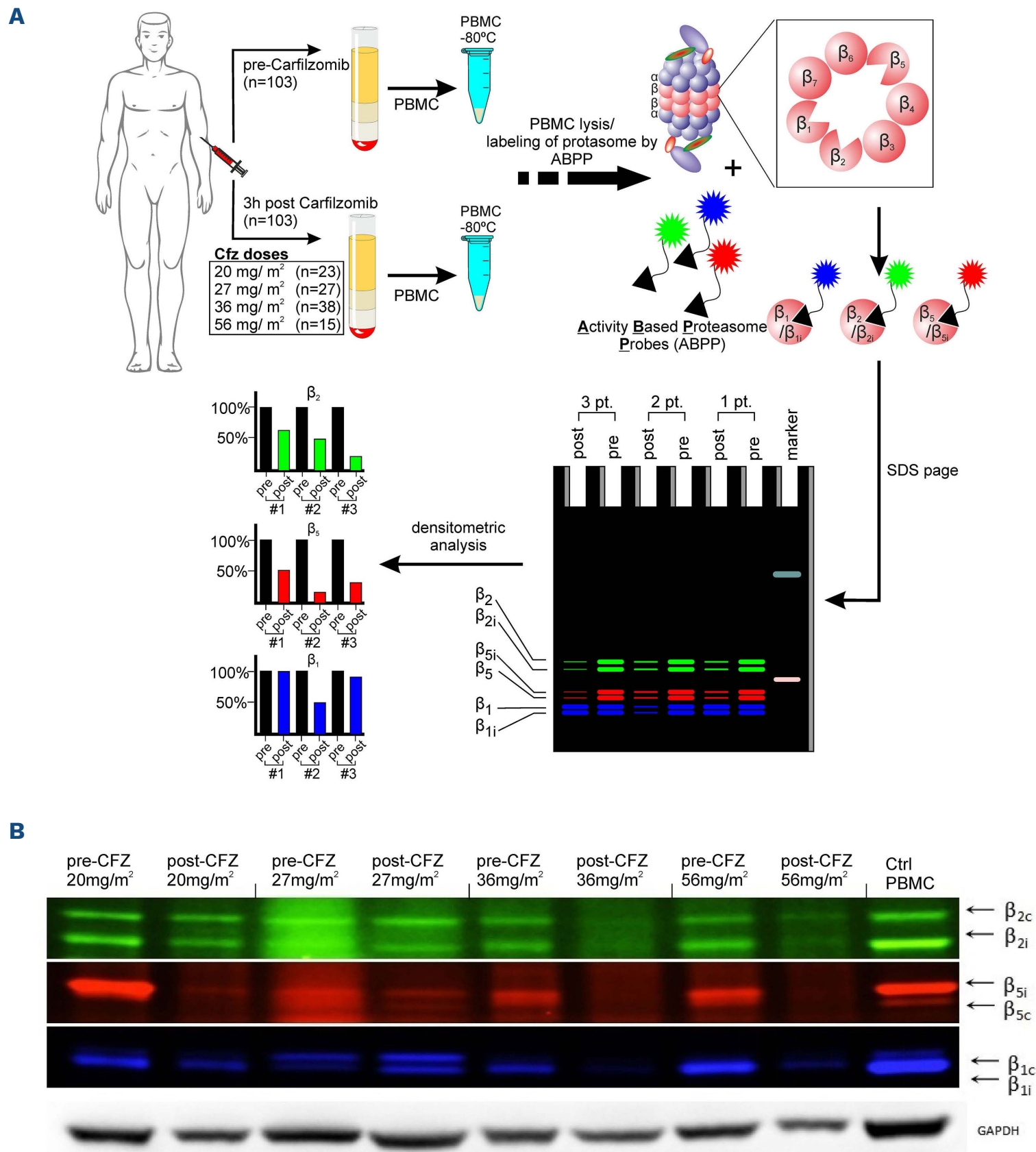
ADC: antibody drug conjugate; BCMA: B-cell maturation antigen; BITE: bispecific T-cell engager; CAR T cell: chimeric antigen modified T cell; CFZ: carfilzomib; EMD: extramedullary disease; IMiDs: immunomodulatory drugs; MM: multiple myeloma; MR: minor response; PD: progressive disease; PI: proteasome inhibitors; PR: partial remission; RR: relapsed/refractory; SCT: stem cell transplant; VGPR: very good partial remission; IgG: immunoglobulin G; <sup>#</sup>defined as presence of at least one of the following: del(17p), amp1q21, t(4;14), t(14;16) and t(14;20); <sup>†</sup>defined as refractory to bortezomib, carfilzomib, lenalidomide, pomalidomide and daratumumab.

with a median residual activity of  $\beta_{2c+i}$  of 81.9% (95% CI: 63.3-104.6) versus 45.5% (95% CI: 26.8-52.8;  $P=0.0001$ ); median residual activity of  $\beta_{1c+i}$  of 92.8% (95% CI: 65.7-127.6) versus 51.0% (95% CI: 39.3-69.5;  $P=0.0005$ ). However, high-dose CFZ did not show significantly superior  $\beta_{5c+i}$  inhibition ( $P>0.05$ ) compared to low-dose CFZ. Taken together, high-dose CFZ demonstrated superior total proteasome inhibition compared to low-dose CFZ through

the co-inhibition of  $\beta_{2c+i}$  and  $\beta_{1c+i}$  proteasome subunits activity with a median residual total proteasome activity of 65.8% (95% CI: 47.7-91.8) versus 35.7% (95% CI 28.2-43.7;  $P=0.0003$ ) (Figure 2A-E).

**High-dose carfilzomib showed more effective anti-multiple myeloma activity than low-dose**

In order to address the issue of whether high-dose CFZ

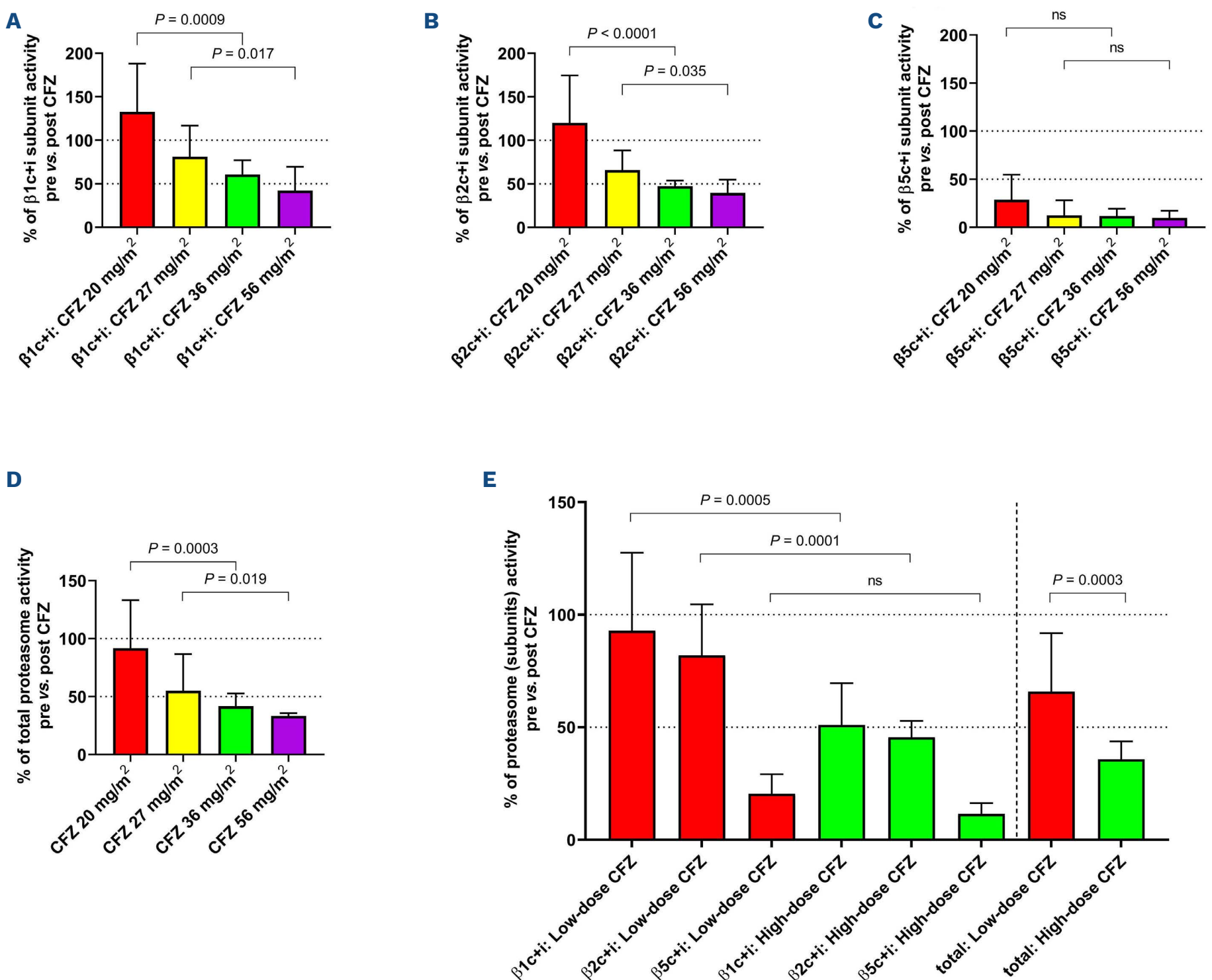


**Figure 1. Experimental design and a representative gel image.** In (A) the experimental design of our study is depicted. We collected 103 paired peripheral blood mononuclear cell (PBMC) samples (before and 3 hours [h] after carfilzomib [CFZ]) from patients with RRMM. CFZ was given at different doses. PBMC were lysed and then proteolytically active constitutive (c) proteasome and immuno (i) proteasome subunits,  $\beta_{1c+i}$ ,  $\beta_{2c+i}$ , and  $\beta_{5c+i}$  were labeled using activity-based proteasome probes. Subsequently, proteins and labeled proteasome subunits were separated with SDS-PAGE, and the  $\beta_{1c+i}$ ,  $\beta_{2c+i}$ , and  $\beta_{5c+i}$  activity was evaluated using densitometric analysis. Panel (B) shows a representative gel (SDS-PAGE) with proteasome subunits activity before and 3 h after CFZ.



could achieve more effective anti-MM efficacy and superior progression-free survival (PFS) compared to low-dose CFZ in routine clinical practice, we investigated the real-world data of 114 RRMM patients who were treated with three currently approved CFZ-containing combinations Kd, KRd, and D-Kd. The median age at therapy initiation was 63 years (range, 39-83 years). In our study, 33, 71, and ten patients received Kd, KRd, and D-Kd combinations, respectively. In total, 20, 11 and five patients were treated with high-dose CFZ in subgroups, Kd, KRd, and D-Kd, respectively. The remaining 78 patients received low-dose CFZ in three different combinations. CFZ and/or LEN dosing was individually determined by the treating physician based on the expected tolerability of each pa-

tient. The median number of prior therapies was two (range, 1-12). Patients received a median of three (range, 1-20) cycles of CFZ combinations. Patient characteristics are summarized in Table 2. The median follow-up time was 15.6 months in this cohort. Overall, 72 patients achieved partial remission (PR) or better, yielding an overall response rate (ORR) of 64.3% in 112 patients with response data (Table 2). Notably, in patients treated with the Kd combination, high-dose CFZ showed a significantly higher ORR compared to low-dose CFZ (ORR: 73.8% vs. 15.4%;  $P=0.003$ ) (Online Supplementary Figure S2). We then analyzed survival outcome in the entire group and found that patients who had received high-dose CFZ showed significantly superior PFS compared to



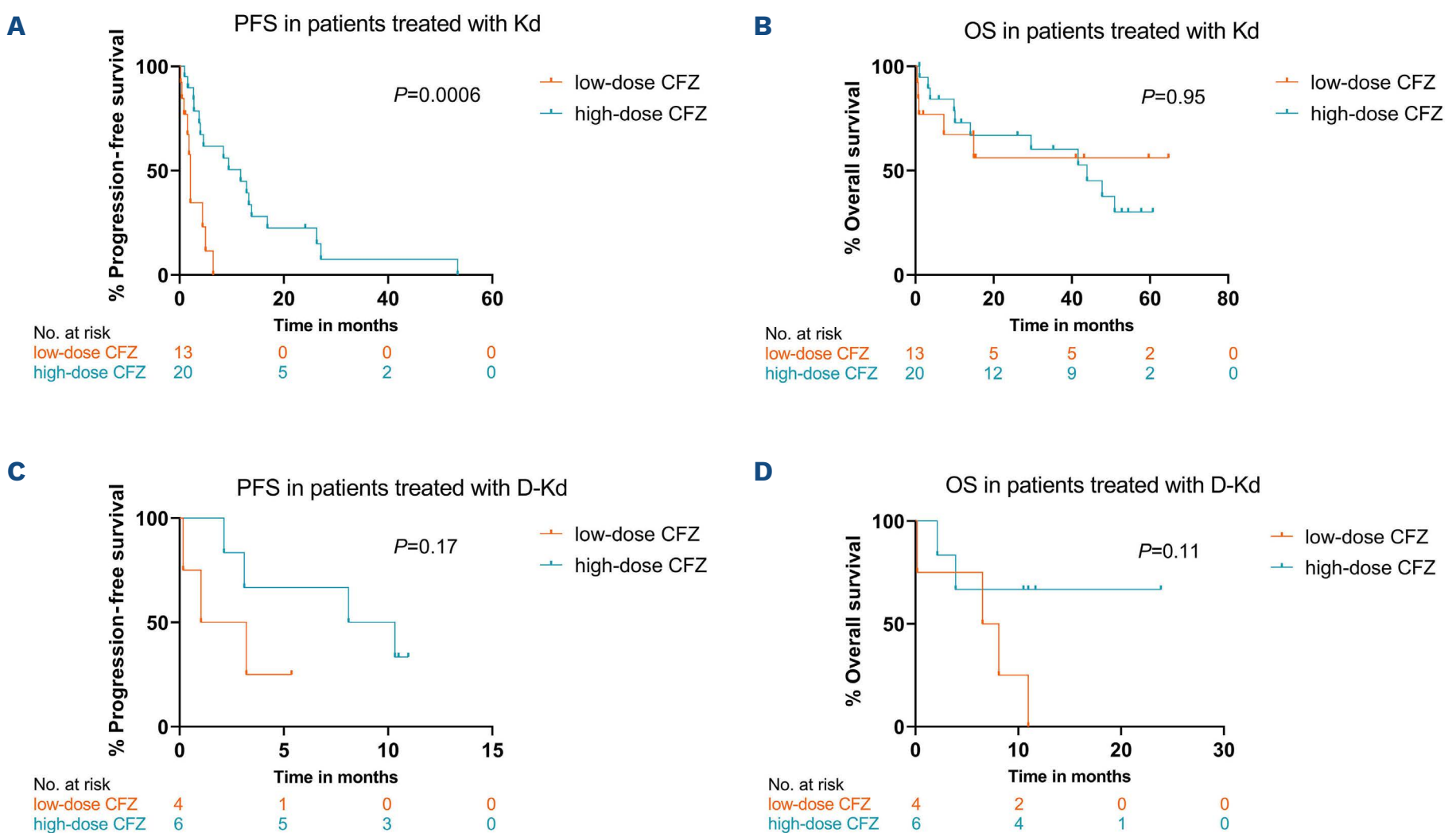
**Figure 2. Proteasome subunit activity evaluated in peripheral blood mononuclear cells of multiple myeloma patients undergoing carfilzomib treatment and their outcome upon dose escalation.** Panels (A-C) display proteasome subunit activity of  $\beta1c+i$ ,  $\beta2c+i$ , and  $\beta5c+i$  pre-carfilzomib (CFZ) vs. 3 hours (h) post CFZ. Panel (D) demonstrates the whole proteasome activity at respective doses prior CFZ vs. 3 h post CFZ treatment. In (E), residual proteasome (subunit) activity 3 h after CFZ infusion is illustrated. ns: not significant;  $P$  value of two-tailed unpaired Student's  $t$ -test. c: constitutive; i: immuno.



low-dose (median PFS: 11.7 vs. 4.5 months;  $P=0.007$ ) (*Online Supplementary Figure S3*). In the subgroup analysis of Kd, high-dose CFZ likewise demonstrated improved PFS over low-dose (median PFS: 11.7 vs. 2.1 months;  $P=0.0006$ ) (Figure 3A, B). In the D-Kd subgroup, we also observed superior PFS in the high-dose CFZ group than in the low-dose CFZ group. However, owing to the low number of cases in this subgroup, the difference in PFS was not statistically significant. (Figure 3C, D). In patients treated with KRD, PFS was significantly longer in patients who had received high-dose CFZ than low-dose (median PFS: 13.2 vs. 5.6 months;  $P=0.02$ ), while LEN dose did not affect PFS in our cohort. (Figure 4). Overall, the most common non-hematologic AE grade  $\geq 3$  included cardiotoxicity ( $n=11$ , 9.6%) and respiratory infections ( $n=11$ , 9.6%). Importantly, among the 11 patients who suffered from cardiotoxicity  $\geq 3$ , only two of them received high-dose CFZ, while the remaining nine patients were treated with low-dose CFZ. Regarding hematologic AE, 29 (25.4%), 34 (29.8%), 37 (32.4%) and 30 (26.3%) patients developed anemia, leukopenia, thrombocytopenia, and neutropenia grade  $\geq 3$ , respectively. Of note, in the entire group, the frequencies of hematologic AE grade  $\geq 3$  were not significantly higher in patients who received high-dose compared to low-dose CFZ (*Online Supplementary Figure S4*).

### Carfilzomib dose escalation recaptured clinical response in relapsed/refractory multiple myeloma patients who were resistant to low-dose carfilzomib

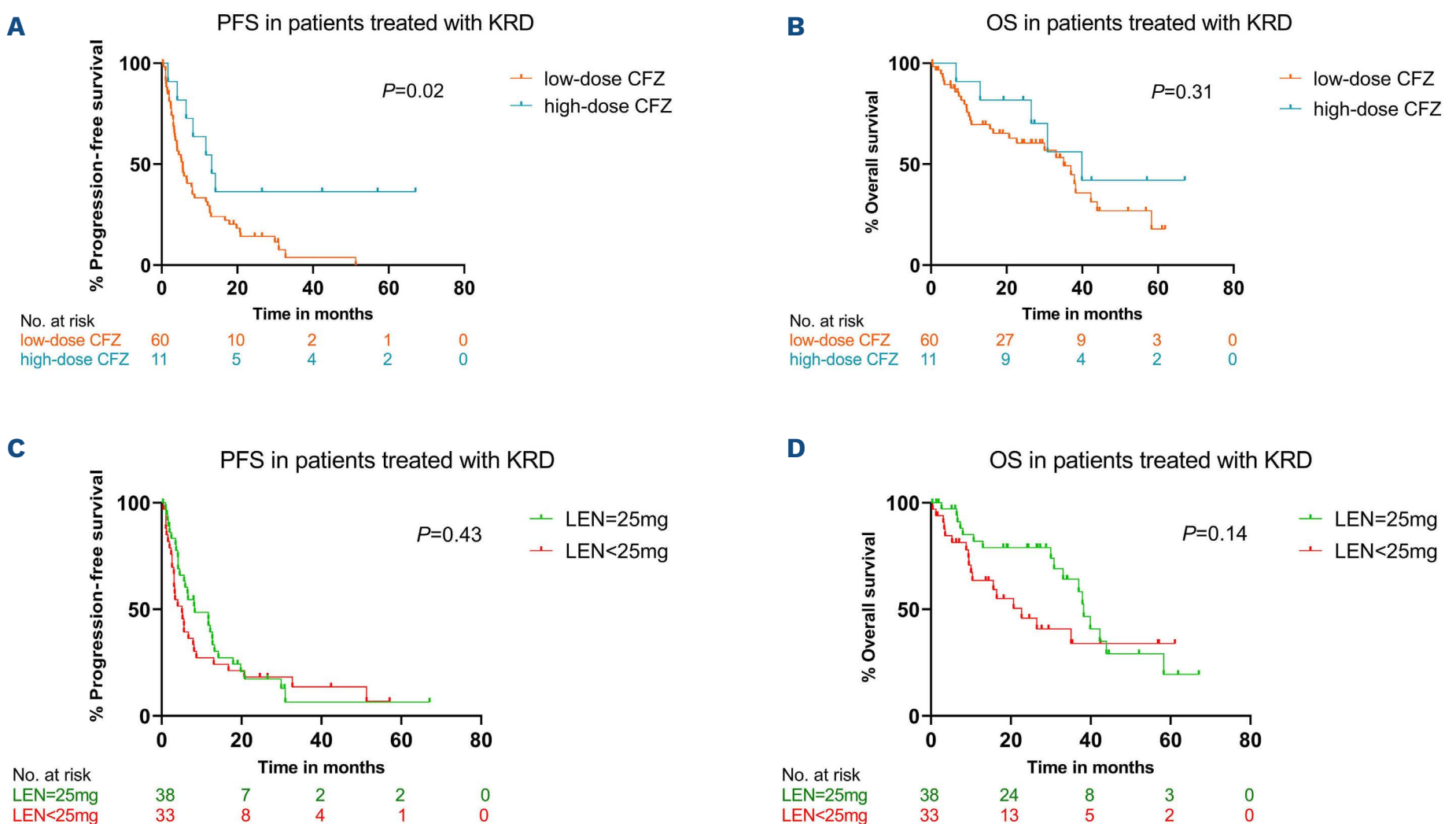
Considering the afore-mentioned findings, we treated 16 patients with RRMM who progressed during low-dose CFZ-containing therapy by escalating CFZ dosing as a personalized treatment approach. The median age of the patients was 71 years (range, 45-83 years), and high-risk cytogenetics was present in 11 (69%) patients. The patients were heavily pretreated with a median of six (range, 2-13) lines of therapies. In prior lines of therapies, all 16 patients received at least one PI, and the vast majority ( $n=15$ , 94%) was pretreated with at least one immunomodulatory drug (IMiD). Daratumumab was administered to 15 (94%) patients in prior treatments. All 16 patients were refractory to their last treatment line. Eleven (69%) patients were refractory to LEN, 13 (81%) to pomalidomide, 14 (88%) to bortezomib (BTZ), eight (50%) to low-dose CFZ, and 15 (94%) patients were refractory to daratumumab in prior lines of therapy. Six (38%) patients were penta-refractory (daratumumab, BTZ, low-dose CFZ, LEN, and pomalidomide). One patient had relapsed MM after treatment with a B-cell maturation antigen (BCMA)-targeted bi-specific antibody, and two patients relapsed after chimeric antigen receptor-modified T-cell (CAR T)



**Figure 3. Survival outcome in multiple myeloma patients that received immunomodulatory imide drug-free carfilzomib-containing regimens: Kd and D-Kd.** Panels demonstrate progression-free survival (PFS) and overall survival (OS) of patients treated with high-dose carfilzomib (CFZ) vs. low-dose CFZ in the group of patients who (A, B) received Kd (carfilzomib, dexamethasone) and (C, D) received D-Kd (carfilzomib, daratumumab, dexamethasone).

therapy. In the current line of therapy, all 16 patients showed progression during low-dose CFZ-containing combination regimens (range of CFZ dose, 15-27 mg/m<sup>2</sup> twice weekly), and six patients presented true extramedullary disease (EMD) without bone contact. Therefore, we escalated the CFZ dose in these patients to high-dose (36 or 56 mg/m<sup>2</sup>), while the doses and schedules of all other anti-MM drugs remained the same (Table 3; *Online Supplementary Table S1*). After a median time to response of 0.7 (range, 0.3-1.1) months, high-dose CFZ recaptured response in nine (56%) patients, including five and four patients with very good partial remission (VGPR) and PR, respectively. Additionally, high-dose CFZ controlled disease progression (minor response) in six (38%) patients, yielding a clinical benefit rate of 94%. Importantly, four of six patients with true EMD achieved a PR (n=2) or VGPR (n=2), and one patient showed a minor response after CFZ dose escalation. This finding underlined that even high-risk RRMM patients with EMD might benefit from CFZ dose escalation. The only patient who progressed after CFZ dose escalation harbored multiple high-risk features, such as high-risk cytogenetics (amp1q21, t(4;14)) and EMD,<sup>17-19</sup> which were potentially associated with aggressive disease and drug resistance, suggesting that CFZ resistance may be related to factors other than CFZ dosing.<sup>20</sup>

Serial PBMC samples before and 3 hours after CFZ administration at different dose levels were evaluated in a patient with CFZ dose escalation (patient #1 in the *Online Supplementary Table S1*). As expected, the  $\beta$ 2c+i proteasome subunit was inhibited more effectively at 36 mg/m<sup>2</sup> (residual  $\beta$ 2c+i activity: 52.8%) than at 20 mg/m<sup>2</sup> (residual  $\beta$ 2c+i activity: 76.5%), whereas the  $\beta$ 5c+i subunit was already meaningfully inhibited at 20 mg/m<sup>2</sup> (residual  $\beta$ 5c+i activity: 29.1%) (*Online Supplementary Figure S5*). After a median follow-up time of 13.0 months, high-dose CFZ achieved a median PFS of 4.4 (95% CI: 4.0-4.8) months and a median overall survival (OS) of 8.9 (95% CI: 6.0-11.7) months in our cohort of patients progressing under low dose CFZ therapy (Figure 5; *Online Supplementary Figure S6*). However, increased doses of CFZ may cause more severe side effects. Indeed, non-hematologic AE grade  $\geq$ 3 were present in six (38%) patients after CFZ dose escalation to high-dose, while only four (25%) patients showed non-hematologic AE grade  $\geq$ 3 during low-dose CFZ-containing treatments. Pneumonia (n=4, 25%) was the most common non-hematologic AE observed after CFZ dose escalation. Interestingly, cardiotoxicity grade  $\geq$ 3 was observed in only one (6%) patient after CFZ dose escalation. However, two (12%) patients experienced cardiotoxicity grade  $\geq$ 3 during the low-dose CFZ phase before dose es-



**Figure 4. Survival outcome in multiple myeloma patients that received immunomodulatory imide drug-containing carfilzomib-containing regimen.** (A) Progression-free survival (PFS) and (B) overall survival (OS) of patients treated with high-dose carfilzomib (CFZ) vs. low-dose CFZ in patients who received KRD (carfilzomib, lenalidomide, dexamethasone). (C, D) PFS and OS of patients who received lenalidomide (LEN) 25 mg vs. < 25 mg.

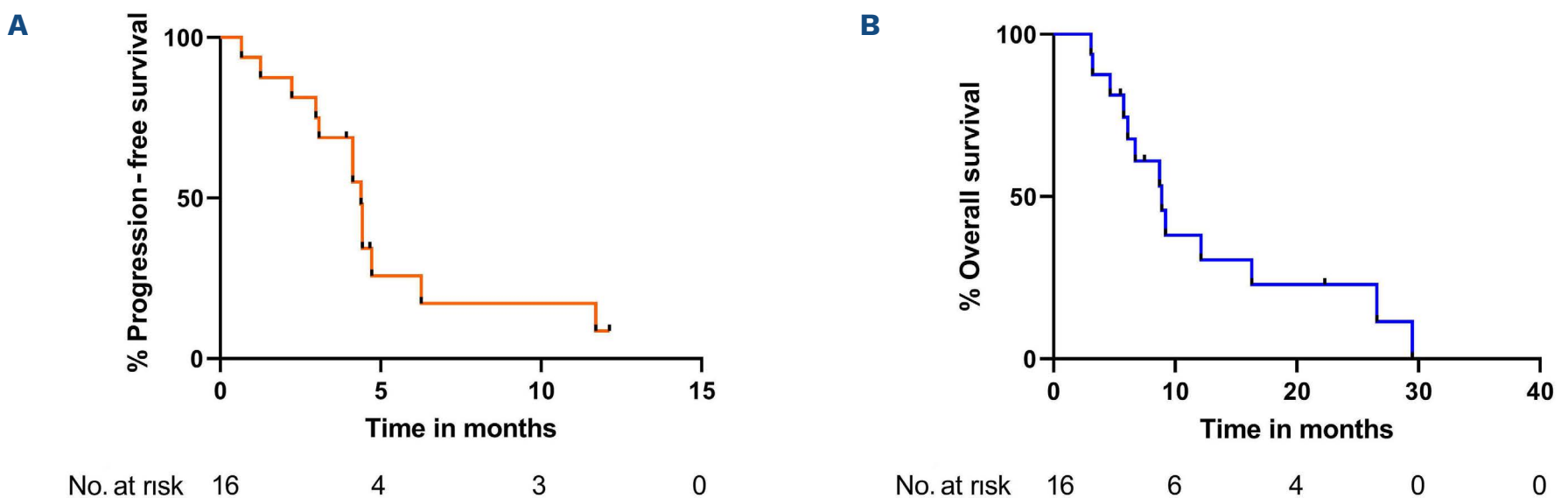
calation, and both patients tolerated high-dose CFZ without cardiotoxicity. Importantly, two patients who achieved PR with high-dose CFZ required CFZ dose reduction to 27 and 20 mg/m<sup>2</sup> due to cardiotoxicity and fatigue, respectively. Notable, both patients showed prompt disease progression after the CFZ dose reduction (*Online Supplementary Table S1*).

## Discussion

In our study, high-dose CFZ showed significantly more effective proteasome inhibition than low-dose CFZ *in vivo* by co-inhibiting the  $\beta$ 2 and  $\beta$ 1 proteasome subunits, suggesting that patients resistant to low-dose CFZ-containing therapies could recapture the clinical response by escalating the CFZ dose. Moreover, high-dose CFZ resulted in longer PFS in RRMM patients treated with CFZ-containing combinations.

In the last few years, CFZ has been evaluated in different dosing regimens within clinical trials. In the phase III ENDEAVOR study evaluating the Kd combination (CFZ 56 mg/m<sup>2</sup> twice weekly), CFZ showed a significantly longer PFS compared to BTZ (median: 18.7 months in the Kd group vs. 9.4 months in the BTZ group) in RRMM patients.<sup>6</sup> Moreover, additional use of daratumumab to the Kd regimen (D-Kd) further improved PFS in RRMM (median: not reached in the D-Kd group vs. 15.8 months in the Kd group), as suggested by the phase III CANDOR trial.<sup>5</sup> In terms of immunomodulatory imide drugs (IMiD)-containing combinations, the KRD regimen demonstrated superior PFS compared with the control group RD (lenalidomide, dexamethasone) (median: 26.3 months in the KRD group vs. 17.6 months in the RD group). However, only low-dose CFZ (27 mg/m<sup>2</sup> twice weekly) was administered to avoid severe AE in the KRD combination.<sup>7</sup> Indeed, at present, the optimal CFZ dose remains controversial in clinical practice.

High-dose once weekly CFZ is being developed as a mainstream regimen to improve patients' compliance with a more convenient proteasome inhibition.<sup>21,22</sup> In the Kd combination, Moreau *et al.* reported that CFZ 70 mg/m<sup>2</sup> once weekly significantly improved PFS and ORR compared with 27 mg/m<sup>2</sup> twice weekly in RRMM.<sup>21</sup> Moreover, in real-world data, high-dose CFZ (56 mg/m<sup>2</sup> twice weekly or 70 mg/m<sup>2</sup> once weekly) likewise showed significantly superior patients survival outcomes when compared to low-dose CFZ (20-27 mg/m<sup>2</sup> twice weekly) in patients treated with Kd combination.<sup>23</sup> In contrast, Ailawadhi *et al.* did not observe a significant ORR or PFS benefit with twice-weekly CFZ 56 mg/m<sup>2</sup> over 27 mg/m<sup>2</sup> in the Kd regimen.<sup>17</sup> Regarding the IMiD-containing CFZ combination, that is, the KRD regimen, our results demonstrate that high-dose CFZ can significantly improve PFS, while the LEN dose does not show any significant impact on patient outcome. Importantly, in different data sets, the safety profile of high-dose CFZ appears similar to that of low-dose CFZ.<sup>17,21,24,25</sup> Here, we provide the first *in vivo* evidence in a clinical setting that the molecular activity of high-dose CFZ ( $\geq 36$  mg/m<sup>2</sup>) differs from that of low-dose CFZ ( $\leq 27$  mg/m<sup>2</sup>) by means of  $\beta$ 2 and  $\beta$ 1 proteasome subunit co-inhibition, which may be a potential mechanism to overcome low-dose drug resistance in RRMM patients. Although the patients in the current study were relatively heterogeneous, including patients treated with Kd, KRD and D-Kd regimens, high-dose CFZ showed improved survival outcome compared with low-dose in the entire cohort as well as in each subgroup Kd, KRD and D-Kd. This is in line with our previous *in vitro* findings<sup>4</sup> and may explain the superior anti-MM activity of higher doses of CFZ compared to lower doses. PI-resistant cells change the level of the  $\beta$ 2 and  $\beta$ 1 proteasome subunits and become insensitive to sole  $\beta$ 5 inhibition. At the same time, co-inhibition of other subunits, especially the  $\beta$ 2 subunit, is able to overcome PI resistance.<sup>4,26,27</sup> However, in our study, there were also patients who did not respond



**Figure 5. Survival outcome in multiple myeloma patients that received carfilzomib dose escalation.** (A) Progression-free survival (PFS) and (B) overall survival (OS) of 16 relapsed/refractory multiple myeloma patients treated with carfilzomib (CFZ) dose escalation.



to CFZ dose-escalation, meaning that high-dose CFZ was not a “game changer” in all patients with heavily pre-treated RRMM. In contrast, disease progression during CFZ-containing treatment may be related to mechanisms other than proteasome inhibition, such as high-risk cytogenetics, EMD, and epigenetic changes.<sup>18,19,28</sup> The underlying resistance mechanisms should be further investigated. In addition, it could not be excluded that low-dose CFZ might potentially achieve a cumulative proteasome inhibition effect on the second day in twice-weekly regimens, and this issue should be addressed in future studies. In recent years, marizomib, a third generation  $\beta$ -lactone- $\gamma$ -lactam PI, has been developed for the treatment of RRMM, and this novel agent is characterized by its irreversible inhibition of all three proteolytic subunits  $\beta$ 5,  $\beta$ 2, and  $\beta$ 1 of the proteasome complex.<sup>29,30</sup> Marizomib has shown high anti-MM activity in RRMM patients and may overcome BTZ and CFZ resistance.<sup>31,32</sup> Therefore, marizomib-containing regimens might be a further option for patients resistant to low-dose CFZ.

Taken together, high-dose CFZ demonstrates superior anti-MM effect to low-dose CFZ by co-inhibiting  $\beta$ 2 and  $\beta$ 1 proteasome subunits, and resistance to low-dose CFZ does not exclude sensitivity to high-dose CFZ. The optimal CFZ dose should be  $\geq 36$  mg/m<sup>2</sup> to achieve sufficient anti-MM activity, while the balance between efficacy and tolerability should be taken into account during treatment decision-making in each patient. In patients with RRMM refractory to low-dose CFZ, dose escalation to  $\geq 36$  mg/m<sup>2</sup> may be worthwhile, as suggested by our data. Our findings provide a rationale for selecting high-dose CFZ to achieve

maximum proteasome inhibition, an optimal anti-MM effect, and to avoid adaptive resistance. The mechanisms of toxicity, particularly cardiotoxicity, of high-dose CFZ remain to be addressed. In addition, future work is required to compare the effects of high-dose CFZ between once-weekly and twice-weekly schedules.

### Disclosures

No conflicts of interest to disclose.

### Contributions

XZ, AB, KMK, CD, LB and LR designed the research. XZ, AB, CV, SN, ET, ES, LH, MML, UM, SH and LB performed the experiments. XZ, JP, MJS, XX, HH, AR, HE, KMK and LR provided patient samples and clinical data. EM, BF and HSO provided the proteasome activity based probes. XZ, AB, CD, LB and LR wrote the manuscript which was approved by all authors and all authors analyzed and interpreted the data.

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### Data-sharing statement

The data generated in this study are available upon request from the corresponding author.

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