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High immunoproteasome and low constitutive proteasome subunit levels correlate with sensitivity to bortezomib-containing chemotherapy: update from the Children's Oncology Group AALL1231 trial

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Authors' contributions

MR, JM, GJa, TH, and JC are responsible for conception and design. MR, JM, SD, ZK, and GJe for administrative support. GJe, DT, TH are responsible for provision of study materials and patients. MR, JM, SD, ZK, GJe, TH, and JC are responsible for collection and assembly of data and MR, JM, SD, ZK, GJa, SZ, GK, TH, and JC for data analyses and interpretation. MR wrote the original draft and MR, JM, SD, ZK, GJe, DT, MD, ML, GJa, SZ, GK, TH, and JC wrote, reviewed and edited the paper. TH was responsible for financial support. All authors are accountable for all aspects of the work and approve the final manuscript.

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Data-sharing statement

Data are available upon request from the corresponding author.

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With current chemotherapy regimens, overall survival (OS) for children and young adults with T-cell acute lymphoblastic leukemia (T-ALL) and precursor B-cell ALL patients are similar.[1] This often requires more intensive upfront therapy since outcomes for relapsed T-ALL patients are still dismal. Therefore, current research focuses on prevention of relapse by improving risk stratification and enhancing response to frontline treatment including novel agents.[2] Based on encouraging results with the proteasome inhibitor (PI) bortezomib (BTZ) in relapsed ALL,[3,4,5] the Children's Oncology Group (COG) conducted a phase III randomized trial (COG-AALL1231) in newly diagnosed T-ALL patients to investigate whether addition of BTZ could improve event-free survival (EFS).[6] Patients were randomized to receive four doses of BTZ during Induction and Delayed Intensification phases (arm-B: aBFM+BTZ) or not (arm-A: aBFM) on an augmented Berlin-Frankfurt-Munster (aBFM) backbone. Previous studies have shown that increased ratios of immunoproteasome (iP), consisting of the catalytic β1i, β2i, and β5i subunits, over constitutive proteasome (cP) subunits (β1, β2, and β5), are associated with BTZ-sensitivity.[7,8,9] A formal correlative objective of the trial was to identify subgroups of patients within COG-AALL1231 that benefited from treatment with BTZ-containing chemotherapy. This study identified increased iP and decreased cP levels being associated with improved survival in patients receiving aBFM+BTZ.

Ninety-nine prospectively collected cryopreserved pre-treatment peripheral blood (PB) samples were available from standard and intermediate risk T-ALL patients enrolled in the COG-AALL1231 after obtaining written informed consent. The study has been performed according to the Declaration of Helsinki and assent, as appropriate, were obtained in accordance with the U.S. National Cancer Institute. The study was approved by the relevant COG committees, CTEP, and the pediatric central institutional review board in accordance with institutional policies for human subjects' research. Patients characteristics are presented in Table 1. We used minimal residual disease (MRD) at end of induction therapy (measured using flow cytometry, cut-off 0.01%), and EFS (relapse and death as events) as treatment response parameters. Of these patients, based on cellular protein yields, 91 samples were available for baseline proteasome subunit expression analysis and 88 samples for proteasome subunit catalytic activity analysis.

We measured the subunit-specific iP proteolytic activity using subunit-specific fluorogenic 7-amino-4-methyl coumarin (AMC) substrates (Ac-ANW-AMC for β5i and Ac-PAL-AMC for β1i)

in protein extracts of PB samples as described previously.[10] The fluorescent reaction product AMC was measured every five minutes over two hours. We calculated the linear slopes based on the reaction product formation depicted as fluorescent units per minute (FU/min). For 51 of 88 samples both β1i and β5i catalytic activities could be measured. A strong correlation was observed between β1i and β5i activity (R=0.95, *P*<0.0001) (Figure S1A). For 37 samples, solely β5i activity was measured due to limitations in patient material.

In previous in vitro studies we reported a downregulation of iP activity in PI-resistant acute leukemia cells,[10] which corresponds to our current finding of lower β5i activity in BTZ-treated MRDpos patients compared to MRDneg patients who were treated with BTZ (median 8.1 vs. 12.4 FU/min, respectively, Mann-Whitney-U *P*=0.02; Figure 1A). While the global β5i-activity was higher in the aBFM arm compared to the aBFM+BTZ arm, this association between β5i activity and MRD was not found in patients who did not receive BTZ (Figure 1B; *P*=NS). In addition, aBFM+BTZ treated patients with high β5i activity (>5 FU/min based on Max-Stat analysis of the BTZ-treatment arm) had a better EFS (4-year EFS: 100%) compared to patients with low β5i activity (4-year EFS: 71.4±17%, Log-rank *P*=0.0012; Figure 1C). β5i activity was not associated with EFS in patients who did not receive BTZ (Figure S1B; *P*=NS). Moreover, the 4-year EFS was significantly better in patients with high iP activity (β5i>5) treated in the aBFM+BTZ arm compared to the aBFM arm (100% versus 78±7% respectively, *P*=0.013; Figure 1D).

Next to iP subunit activity, we also determined proteasome subunit expression levels since previous studies reported a downregulation of iP and an upregulation of cP subunit expression in PI-resistant acute leukemia cells.[7,11] Since β5(i) and β1(i) constitute the primary subunits targeted by BTZ, this current study focusses on those subunits. We determined baseline proteasome subunit expression (iP and cP) by Western blot as described previously.[8] 15 out of 91 samples, with sufficient protein yields, were excluded due to insufficient β-actin signal (<2x background). The 76 samples included in our analyses were normalized to β-actin and calculated relative to the subunit expression of a human T-ALL cell line CCRF-CEM (CEM-WT) protein sample to correct for variation between blots. aBFM+BTZ treated patients with low cP expression (β1<1.2 or β5<0.6) demonstrated a 4-year EFS of 100%, which was significantly better compared to patients with high β1 (79±13%, *P*=0.0038) or high β5 (83±11%, *P*=0.012) expression (Figure 2A-B).

In previous relapsed ALL COG-AALL07P1 and AML COG-AAML07P1 trials,[8,9] the ratios of iP/cP expression correlated with *ex vivo* PI-sensitivity and were an indicator of sensitivity to BTZ-containing re-induction chemotherapy. This was also found in the current study by an improved EFS in patients with high iP/cP ratios (P=0.003 for β1i/β1-ratio and P=0.00013 for β5i/β5-ratio, Figure S2A-B), with 2 of 4 of the patients with low β5i/β5-ratio having an event, versus only 1 of 26 of the patients with a high β5i/β5-ratio. Of note, this ratio was mainly determined by the cP expression for β5, since β5i expression was relatively low in all patient samples (Figure S2E).

Patients in the aBFM+BTZ arm with low cP expression (β1<1.2; β5<0.6) had a significantly better 4-year EFS (100%) compared to patients with low cP expression in the aBFM arm without BTZ-treatment (β1<1.2; EFS 72±11%, P=0.010 or β5<0.6; EFS 69±12%, P=0.012, Figure 2C-D). Moreover, patients with high β1i/β1-ratio treated in the aBFM+BTZ arm demonstrated an improved EFS compared to patients in the aBFM arm without BTZ-treatment (P=0.013 Figure S2C). For the β5i/β5-ratio this did not reach statistical significance (P=0.058 Figure S2D) probably due to the late event after 4 years (off-therapy death). Overall, these data of proteasome subunits expressions indicates that patients with low cP expression (and thereby high iP/cP ratios) in the aBFM+BTZ arm benefited from the addition of BTZ.

Since mechanistically, suppression of NFκB activation can increase response to PIs,[3] we assessed NFκB inhibition in 45 samples both before therapy and 24-hours post BTZ-administration by ELISA assay, as described previously.[3] Indeed, a significant suppression of NFκB activity was noted in the aBFM+BTZ arm, and not in the aBFM arm (Figure S3).

While there was no statistically significant benefit from BTZ-containing therapy in the overall COG-AALL1231 T-ALL trial cohort,[6] this study identified a subgroup of patients with low cP and high iP that benefitted from BTZ. Our proteasome subunit expression data and activity analysis in this newly diagnosed T-ALL pediatric patients treated on COG-AALL1231 confirm the results of previous studies that high iP (activity and expression) and low cP (expression) correlated with better BTZ-response.[8,9] Moreover, MRDneg BTZ patients also demonstrated significantly higher baseline β5i activity compared to MRDpos patients. Lastly, patients with low cP expression and high iP/cP ratio's demonstrated better EFS. From this perspective, it is

interesting to speculate whether patients with high iP activity, low cP expression, and high iP/cP ratios from the non-BTZ aBFM arm might have benefited from BTZ-treatment realizing that these patients had a significantly worse EFS compared to patients receiving BTZ. Interestingly, COG-AALL1231 T-cell lymphoma cohort patients showed an improvement in EFS and OS for BTZ-containing therapy[6] and is now considered standard of care in some countries. For T-cell lymphoma research, proteasome subunits are currently being investigated to potentially distinguish responders from non-responders.

This study is based on PB-samples, where blast percentages could vary. While blast percentages where equally distributed (Table 1), as an additional check we re-analyzed the data to see whether lower blast percentages in some of the patient samples may have influenced the results, which did not prove to be the case.

Limitations of this study are the relatively low number of patient samples and the limited sensitivity of iP protein expression measurements. The semi-quantitative Western blot measurements of proteasome subunits required internal controls such as β-actin and a reference cell line to correct for inter-blot differences, and activity experiments rely on accurate protein concentration measurements. Therefore, other quantitative techniques, such as ProCISE or activity-based probes, to accurately quantify the fraction of cP and iP subunits, are probably more suitable for implementation in a prospective clinical setting.[9,12,13] In our study, we could not use ProCISE as this technique requires large cell numbers, for which our samples sizes were too small. Based on our study we cannot conclude whether the measured subunits were possibly part of hybrid proteasomes, which have been described to occur.[7,14]

Despite these limitations, there are various mechanistic studies supporting our findings, e.g. β5i-knockdown diminishes PI-sensitivity, while by interferon-γ upregulation of β5i or β5-knockdown increases sensitivity.[15,16] Moreover, inhibition of normal function of iP e.g. blocks presentation of MHC class I ligands, provokes accumulation of harmful protein aggregates and impairs activation of NFκB, the transcription factor for pro-survival genes and inflammatory cytokines.[17] In this study, suppression of NFκB activity was observed within 24-hours after BTZ-administration.

Together, our results confirm that high iP and low cP correlate with BTZ-response and patients from the COG-AALL1231 trial harboring these subunit levels benefitted from BTZ-containing therapy. Individualized treatment selection based on (i)P subunit data may show added value of BTZ for these well-defined subsets of T-ALL patients.

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TABLE 1. Patient Characteristics (n=99)

Characteristic	aBFM (arm A) n = 52	aBFM+BTZ (arm B) n = 47	*P*-value[1]
Age, years, No. (%)			0.9
< 10	21 (40)	17 (36)	
10-16	22 (42)	20 (43)	
> 16	9 (17)	10 (21)	
Sex, No. (%)			0.042
Male	46 (88)	34 (72)	
Female	6 (12)	13 (28)	
WBC (X 1,000/µL), No. (%)			>0.9
< 50	5 (10)	5 (11)	
≥ 50	47 (90)	42 (89)	
Risk Group, No. (%)			0.6
Intermediate risk	37 (71)	31 (66)	
Standard risk	15 (29)	16 (34)	
MRD EOI, No. (%)			0.094
MRD < 0.01%	28 (54)	33 (70)	
MRD ≥0.01%	24 (46)	14 (30)	
MRD EOC, No. (%)			>0.9
MRD < 0.01%	23 (92)	13 (93)	
MRD ≥0.01%	2 (8)	1 (7)	
Blasts %, Median (Q1, Q3)			
Blasts % PAA	85 (79, 89)	85 (85, 90)	0.5
Blasts % WB	85 (85, 89)	85 (85, 90)	0.8

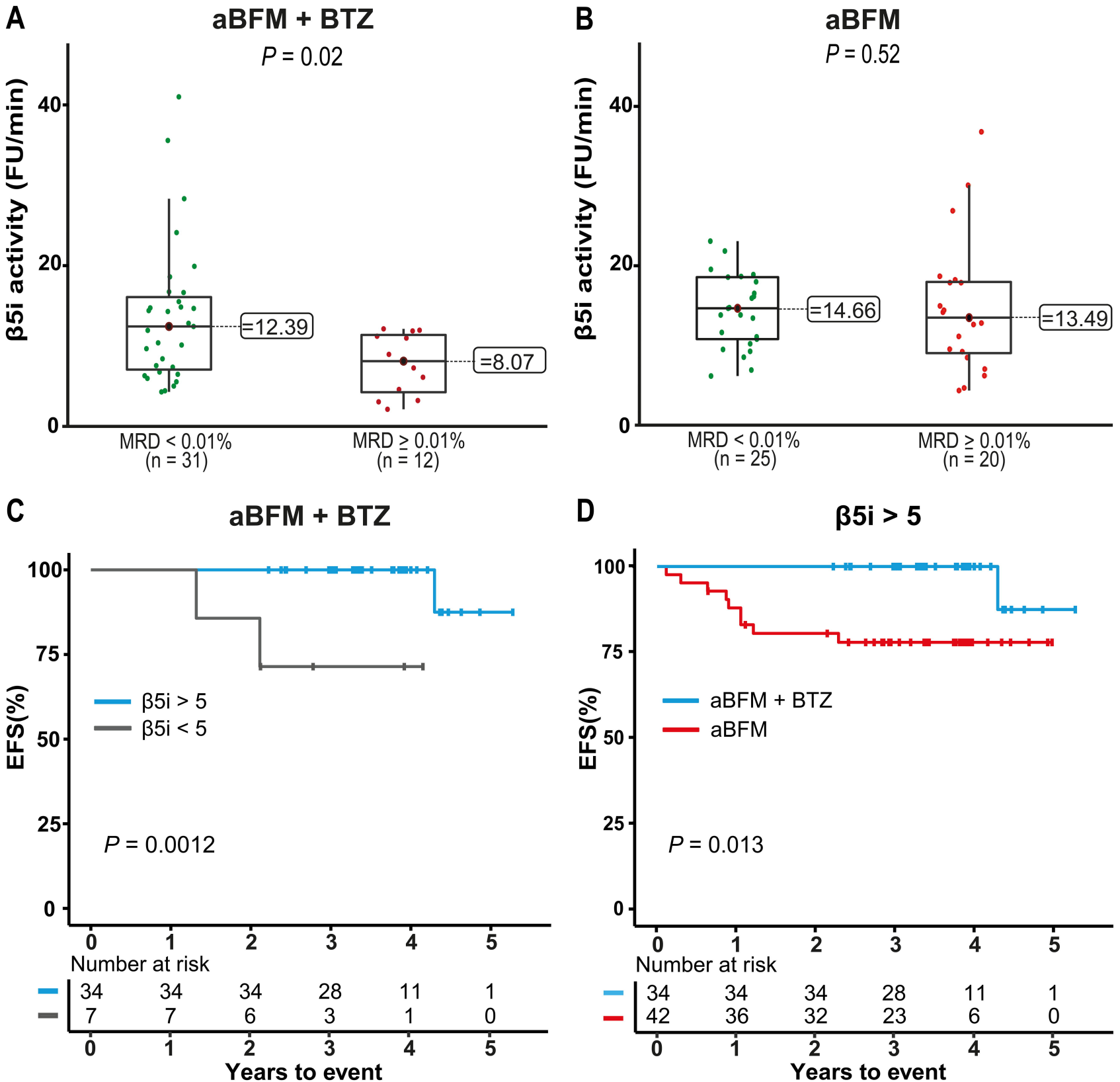
[1] Person's Chi-squared test: Fisher's exact test: Wilcoxon rank sum test

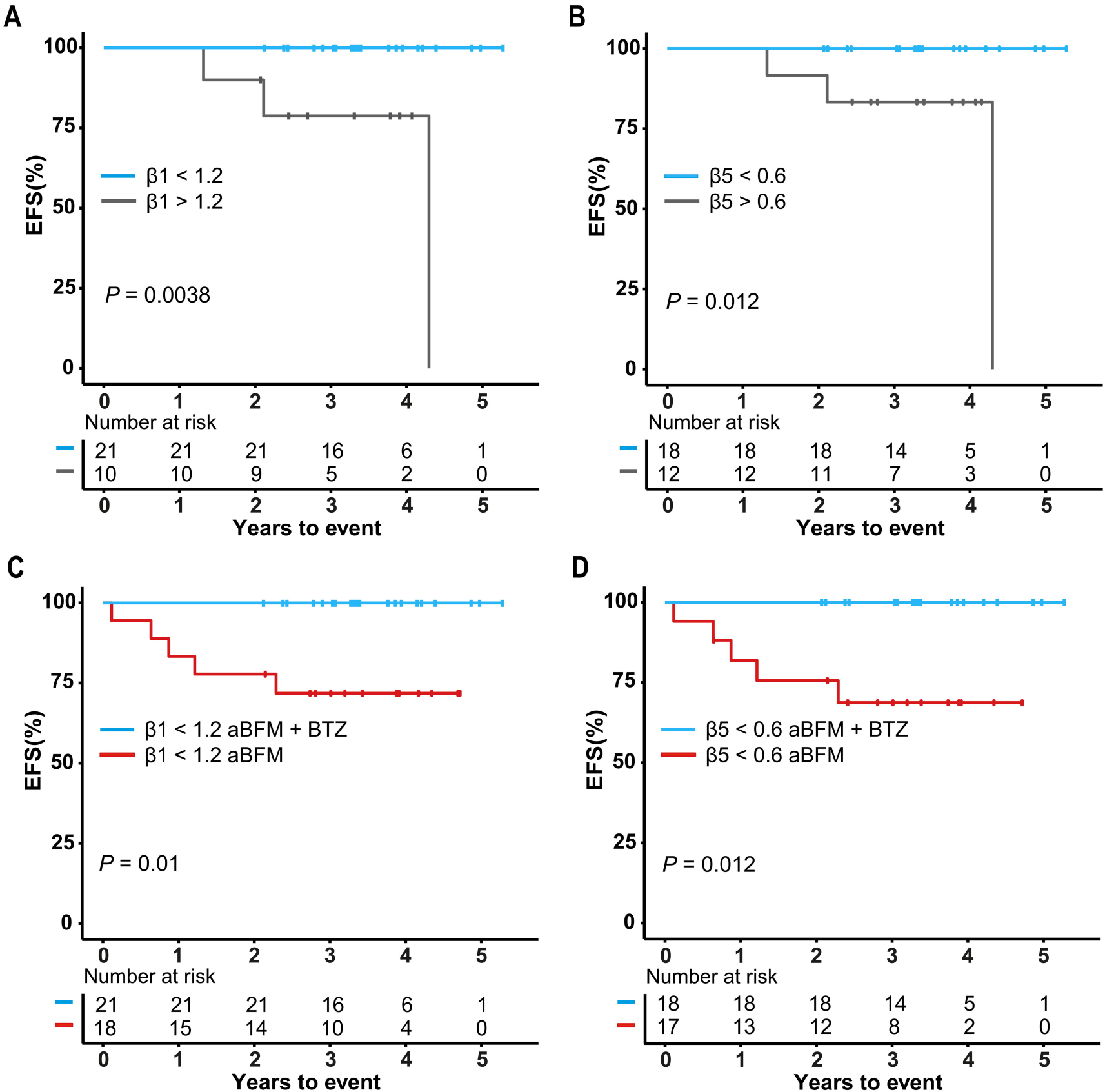
Abbreviations: aBFM, augmented Berlin-Frankfurt-Munster; BTZ, bortezomib; WBC, white blood cell count; MRD, minimal residual disease as measured by flow cytometry; EOI, end of induction; EOC, end of consolidation; PAA, proteasome activity assay; WB, Western Blot.

Figure legends

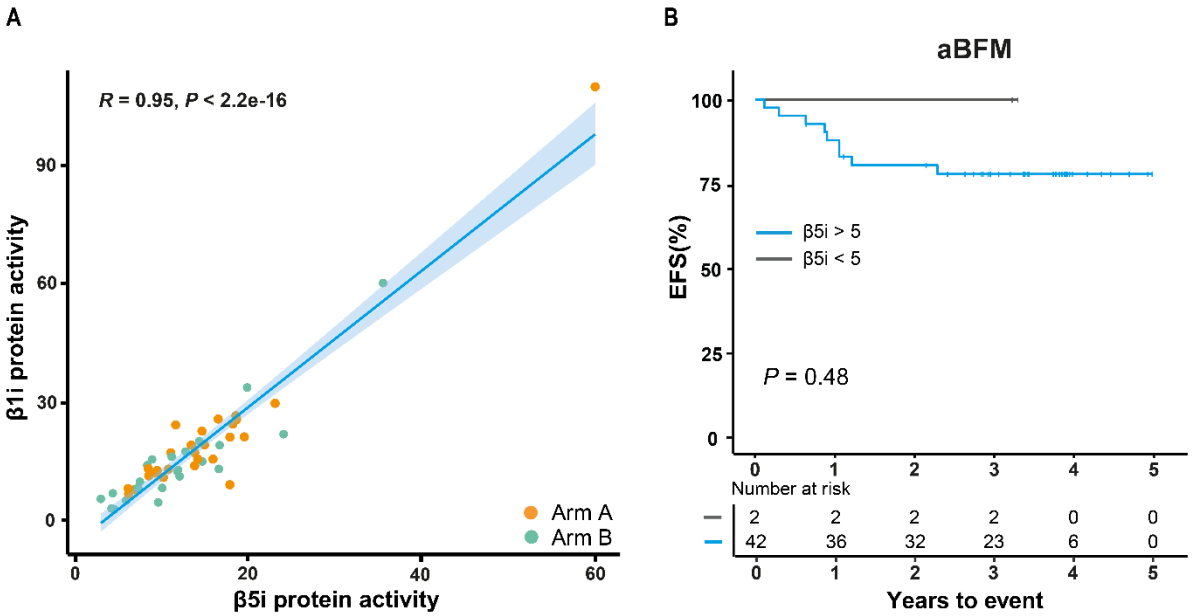
Figure 1. Baseline β5i activity in primary T-cell acute lymphoblastic leukemia patient samples. (A,B) On the y-axis β5i activity is shown as fluorescent units/minute (FU/min). Differences are depicted between minimal residual disease (MRD), MRDneg (<0.01%, green) and MRDpos (≥0.01%, red), after end of induction in **(A)** patients receiving augmented Berlin-Frankfurt-Munster (aBFM) + bortezomib (BTZ) therapy and **(B)** patients receiving aBFM alone. **(C,D)** Event free survival (EFS) in patients **(C)** receiving aBFM+BTZ stratified by β5i activity **(D)** patients with a high β5i (> 5 FU/min), stratified by treatment arm (aBFM+BTZ vs aBFM). *The late event after 4 years is not a relapse, but off-therapy death*.

Figure 2. Proteasome subunit expression and event free survival. **(A-B)** Event free survival (EFS) in patients of the augmented Berlin-Frankfurt-Munster + bortezomib (aBFM+BTZ) group stratified by low vs high subunit expression **(A)** β1 expression, **(B)** β5 expression. *The late event after 4 years is not a relapse, but off-therapy death*. **(C-D)** EFS in patients with low constitutive proteasome (cP) expression stratified by treatment arm for patients with **(C)** β1 expression <1.2, and **(D)** β5 expression <0.6.

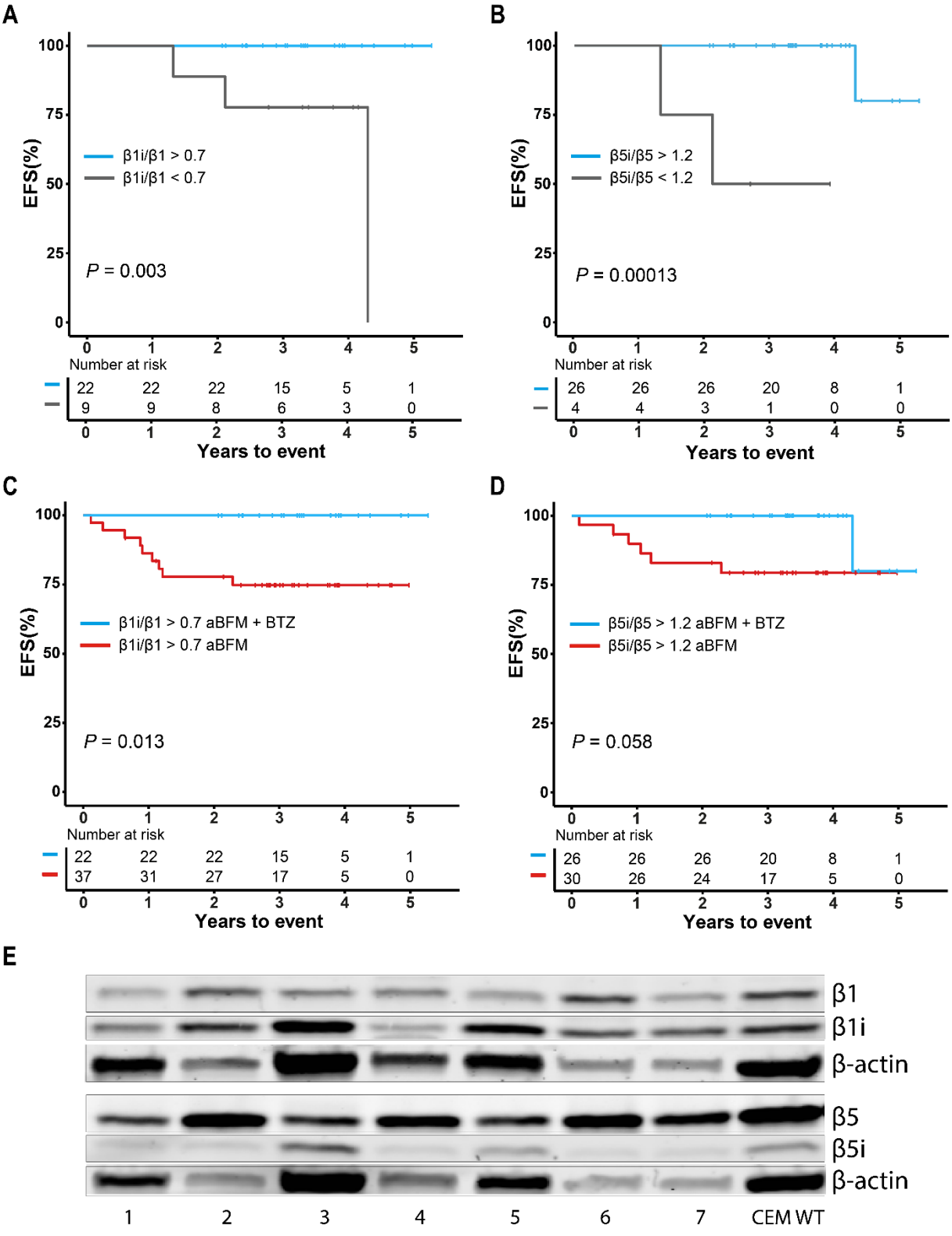

A
aBFM + BTZ
P = 0.02
β5i activity (FU/min)
40
20
0
=12.39
=8.07
MRD < 0.01%
(n = 31)
MRD ≥ 0.01%
(n = 12)
B
aBFM
P = 0.52
β5i activity (FU/min)
40
20
0
=14.66
=13.49
MRD < 0.01%
(n = 25)
MRD ≥ 0.01%
(n = 20)
C
aBFM + BTZ
EFS(%)
100
75
50
25
0
β5i > 5
β5i < 5
P = 0.0012
0 1 2 3 4 5
Number at risk
34 34 34 28 11 1
7 7 6 3 1 0
0 1 2 3 4 5
Years to event
D
β5i > 5
EFS(%)
100
75
50
25
0
aBFM + BTZ
aBFM
P = 0.013
0 1 2 3 4 5
Number at risk
34 34 34 28 11 1
42 36 32 23 6 0
0 1 2 3 4 5
Years to event


A
100
75
50
25
0
EFS(%)
β1 < 1.2
β1 > 1.2
P = 0.0038
0 1 2 3 4 5
Number at risk
21 21 21 16 6 1
10 10 9 5 2 0
0 1 2 3 4 5
Years to event
B
100
75
50
25
0
EFS(%)
β5 < 0.6
β5 > 0.6
P = 0.012
0 1 2 3 4 5
Number at risk
18 18 18 14 5 1
12 12 11 7 3 0
0 1 2 3 4 5
Years to event
C
100
75
50
25
0
EFS(%)
β1 < 1.2 aBFM + BTZ
β1 < 1.2 aBFM
P = 0.01
0 1 2 3 4 5
Number at risk
21 21 21 16 6 1
18 15 14 10 4 0
0 1 2 3 4 5
Years to event
D
100
75
50
25
0
EFS(%)
β5 < 0.6 aBFM + BTZ
β5 < 0.6 aBFM
P = 0.012
0 1 2 3 4 5
Number at risk
18 18 18 14 5 1
17 13 12 8 2 0
0 1 2 3 4 5
Years to event

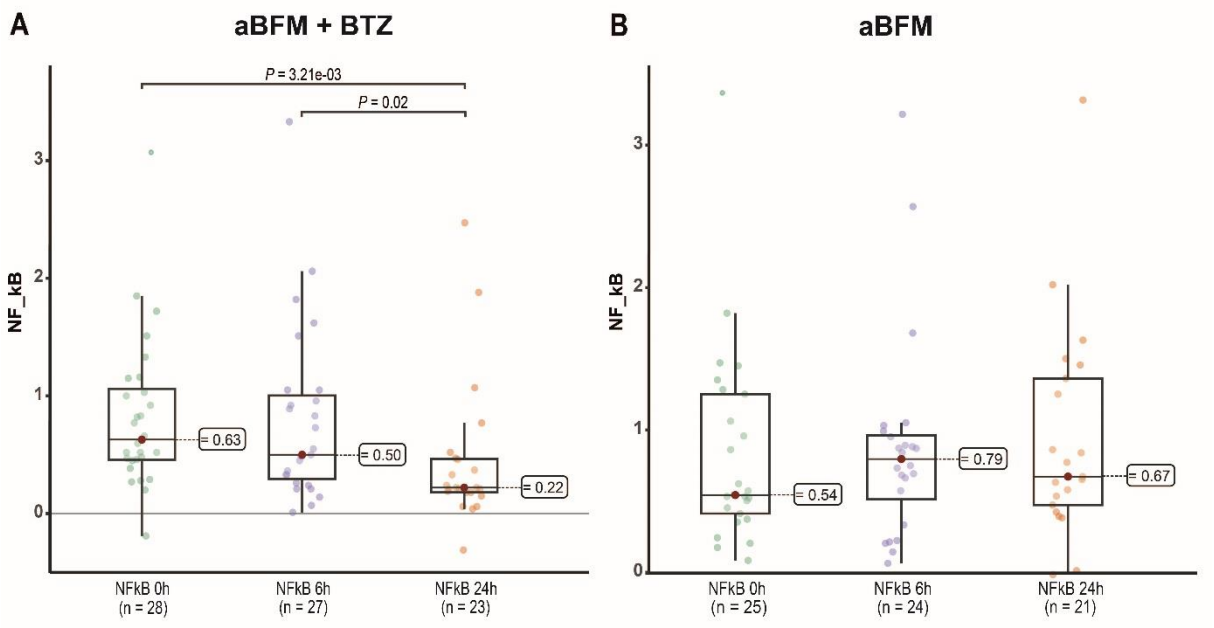
DATA SUPPLEMENT



Supplemental Figure S1. Proteasome subunit catalytic activity analysis. (A) Correlation between baseline β1i- and β5i-associated catalytic activity, measuring slopes as arbitrary fluorescent units/minute (FU/min), in protein extracts of primary T-ALL patients. **(B)** EFS for patients stratified by β5i activity in patients not receiving BTZ.



Supplemental Figure S2 Proteasome subunit expression analysis. (A-B) EFS in patients receiving BTZ stratified by subunit expression ratio's **(A)** β1i/β1 expression ratio, and **(B)** β5i/β5 expression ratio. **(C-D)** EFS stratified by treatment arm for patients with **(C)** high β1i/β1 expression ratio, and **(D)** high β5i/β5 expression ratio. **(E)** Representative blot of Western Blot analysis of cP and iP expression in seven patient samples and one internal control (BTZ-sensitive T-ALL cell line CEM/WT).



Supplemental Figure S3 NFkB-activity. NFkB-activity before treatment (0h), at 6 hours and 24 hours after **(A)** aBFM+BTZ treatment, **(B)** aBFM treatment. Number in boxes depict median.