

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

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; *clinicaltrials.gov*. Identifier: NCT02112916) 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 $\beta 1i$, $\beta 2i$, and $\beta 5i$ subunits, over constitutive proteasome (cP) subunits ($\beta 1$, $\beta 2$, and $\beta 5$), 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 US 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, cutoff 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 $\beta 5i$ and Ac-PAL-AMC for $\beta 1i$) in protein extracts of PB samples as described previously.¹⁰ The fluorescent reaction product AMC was measured every 5 minutes over 2 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 $\beta 1i$ and $\beta 5i$ catalytic activities could be measured. A strong correlation was observed between $\beta 1i$ and $\beta 5i$ activity ($R=0.95$; $P<0.0001$) (*Online Supplementary Figure S1A*). For 37 samples, solely $\beta 5i$ 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 $\beta 5i$ activity in BTZ-treated MRD-positive patients compared to MRD-negative 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 $\beta 5i$ -activity was higher in the aBFM arm compared to the aBFM+BTZ arm, this association between $\beta 5i$ activity and MRD was not found in patients who did not receive BTZ (Figure 1B; $P=\text{not significant [NS]}$). In addition, aBFM+BTZ treated patients with high $\beta 5i$ 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 $\beta 5i$ activity (4-year EFS: $71.4\pm 17\%$, log-rank $P=0.0012$; Figure 1C). $\beta 5i$ activity was not associated with EFS in patients who did not receive BTZ (*Online Supplementary Figure S1B*; $P=\text{NS}$). Moreover, the 4-year EFS was significantly better in patients with high iP activity ($\beta 5i>5$) treated in the aBFM+BTZ arm compared to the aBFM arm (100% vs. $78\pm 7\%$ 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 $\beta 5(i)$ and $\beta 1(i)$ constitute the primary subunits targeted by BTZ, this current study focuses on those subunits. We determined baseline proteasome subunit expression (iP and cP) by western blot as described previously.⁸ Fifteen of 91 samples, with sufficient protein yields, were excluded due to insufficient β -actin signal ($<2\times$ background). The 76

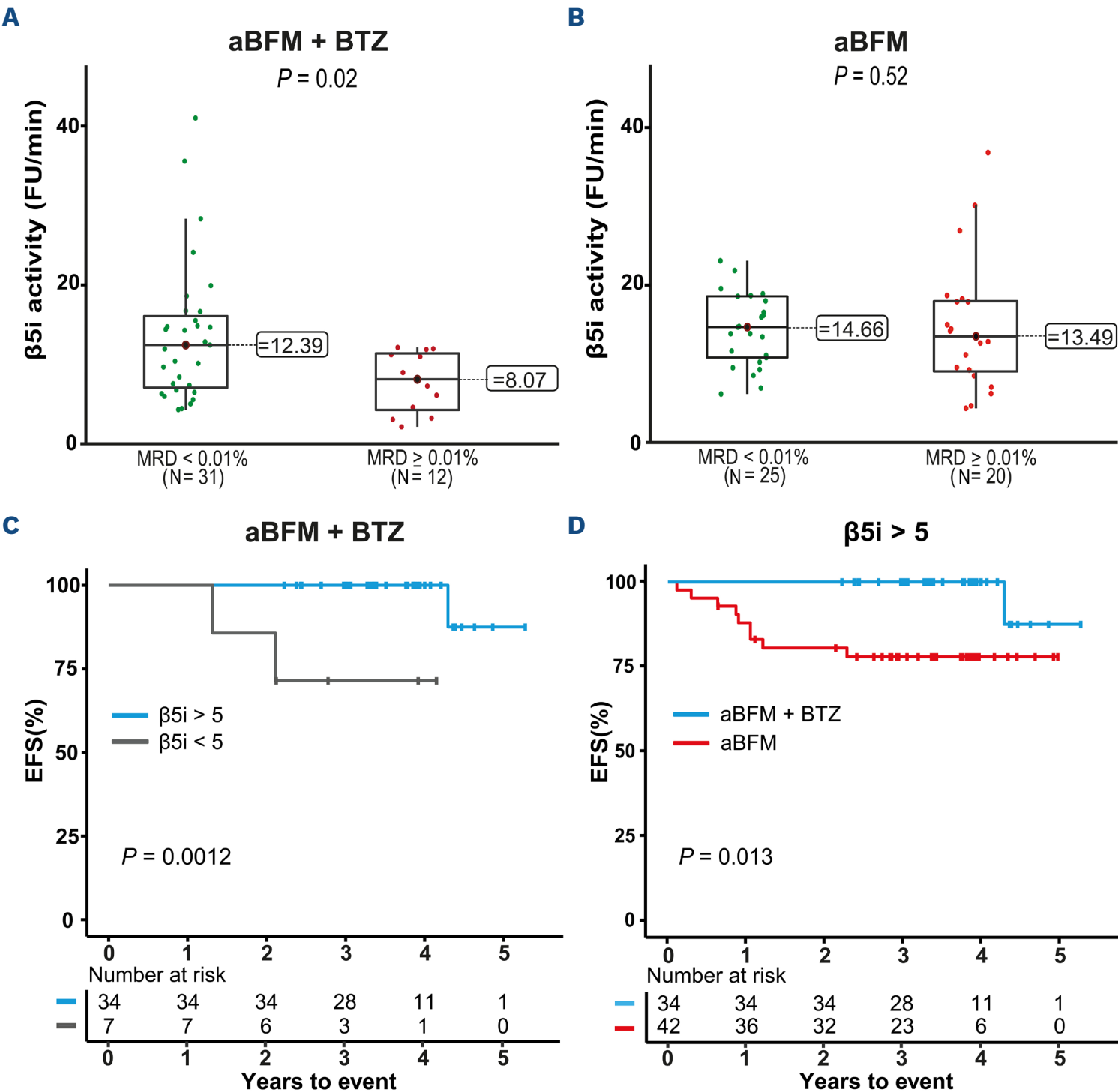


Figure 1. Baseline $\beta 5i$ activity in primary T-cell acute lymphoblastic leukemia patient samples. (A, B) On the y-axis $\beta 5i$ activity is shown as fluorescent units/minute (FU/min). Differences are depicted between minimal residual disease (MRD), MRD-negative (<0.01%, green) and MRD-positive (\geq 0.01%, red), after end of induction in (A) patients receiving augmented Berlin-Frankfurt-Münster plus bortezomib (aBFM+BTZ) therapy and (B) patients receiving aBFM alone. (C, D) Event-free survival (EFS) in patients (C) receiving aBFM+BTZ stratified by $\beta 5i$ activity (D) patients with a high $\beta 5i$ (> 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.

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-wild-type) protein sample to correct for variation between blots. aBFM+BTZ treated patients with low cP expression ($\beta 1 < 1.2$ or $\beta 5 < 0.6$) demonstrated a 4-year EFS of 100%, which was significantly better compared to patients with high $\beta 1$ ($79 \pm 13\%$; $P = 0.0038$) or high $\beta 5$ ($83 \pm 11\%$; $P = 0.012$) 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 $\beta 1i/\beta 1$ -ratio and $P = 0.00013$ for $\beta 5i/\beta 5$ -ratio; *Online Supplementary Figure S2A, B*), with two of four of the patients with low $\beta 5i/\beta 5$ -ratio having an event, *versus* only one of 26 of the patients with a high $\beta 5i/\beta 5$ -ratio. Of note, this ratio was mainly determined by the cP expression for $\beta 5$, since $\beta 5i$ expression was relatively low in all patient samples (*Online Supplementary Figure S2E*). Patients in the aBFM+BTZ arm with low cP expression ($\beta 1 < 1.2$; $\beta 5 < 0.6$) had a significantly better 4-year EFS (100%) compared to patients with low cP expression in the aBFM arm without BTZ-treatment ($\beta 1 < 1.2$; EFS $72 \pm 11\%$; $P = 0.010$; or $\beta 5 < 0.6$; EFS $69 \pm 12\%$; $P = 0.012$; Figure 2C, D). Moreover,

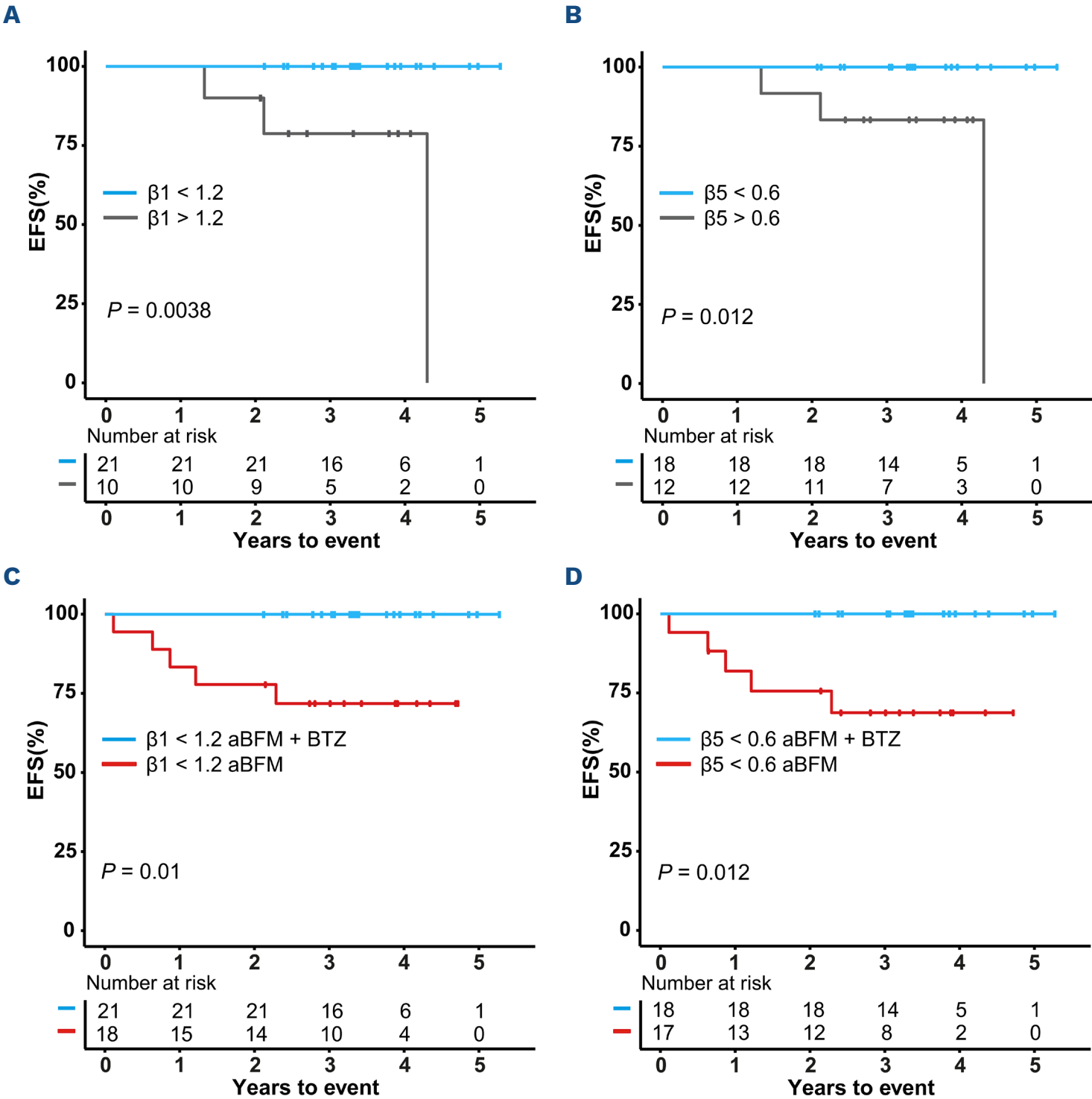


Figure 2. Proteasome subunit expression and event-free survival. (A, B) Event-free survival (EFS) in patients of the augmented Berlin-Frankfurt-Munster plus bortezomib (aBFM+BTZ) group stratified by low *versus* high subunit expression: (A) $\beta 1$ expression, (B) $\beta 5$ expression. The late event 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) $\beta 1$ expression <1.2, and (D) $\beta 5$ expression <0.6.

patients with high $\beta 1i/\beta 1$ -ratio treated in the aBFM+BTZ arm demonstrated an improved EFS compared to patients in the aBFM arm without BTZ-treatment ($P=0.013$; *Online Supplementary Figure S2C*). For the $\beta 5i/\beta 5$ -ratio this did not reach statistical significance ($P=0.058$; *Online Supplementary 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 benefitted from the addition of BTZ. Since mechanistically suppression of NF κ B activation can increase response to PI,³ we assessed NF κ B inhibition in 45 samples both before therapy and 24 hours post BTZ-administration by enzyme-linked immunosorbant assay, as

described previously.³ Indeed, a significant suppression of NF κ B activity was noted in the aBFM+BTZ arm, and not in the aBFM arm (*Online Supplementary 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, MRD-negative BTZ patients also demonstrated significantly higher baseline $\beta 5i$ activity compared to MRD-positive pa-

tients. 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 therapy⁶ 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 were 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

Table 1. Patient characteristics (N=99).

| Characteristic | aBFM (arm A) N=52 | aBFM+BTZ (arm B) N=47 | P ¹ |
|--------------------------------|----------------------|--------------------------|----------------|
| Age in years, N (%) | | | 0.9 |
| <10 | 21 (40) | 17 (36) | |
| 10-16 | 22 (42) | 20 (43) | |
| >16 | 9 (17) | 10 (21) | |
| Sex, N (%) | | | 0.042 |
| Male | 46 (88) | 34 (72) | |
| Female | 6 (12) | 13 (28) | |
| WBC x10 ⁹ /L, N (%) | | | >0.9 |
| <50 | 5 (10) | 5 (11) | |
| ≥50 | 47 (90) | 42 (89) | |
| Risk group, N (%) | | | 0.6 |
| Intermediate risk | 37 (71) | 31 (66) | |
| Standard risk | 15 (29) | 16 (34) | |
| MRD EOI, N (%) | | | 0.094 |
| <0.01% | 28 (54) | 33 (70) | |
| ≥0.01% | 24 (46) | 14 (30) | |
| MRD EOC, N (%) | | | >0.9 |
| <0.01% | 23 (92) | 13 (93) | |
| ≥0.01% | 2 (8) | 1 (7) | |
| Blasts %, median (Q1, Q3) | | | |
| PAA | 85 (79, 89) | 85 (85, 90) | 0.5 |
| WB | 85 (85, 89) | 85 (85, 90) | 0.8 |

¹Person's χ^2 test, Fisher's exact test, Wilcoxon rank sum test. 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; Q1, Q3: quartile1, quartile 3; PAA: proteasome activity assay; WB: western blot.

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., β 5i-knockdown diminishes PI-sensitivity, while by interferon- γ upregulation of β 5i or β 5-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 NF κ B, the transcription factor for pro-survival genes and inflammatory cytokines.¹⁷ In this study, suppression of NF κ B 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 benefited from BTZ-containing therapy. Individualized treatment selection based on iP subunit data may show added value of BTZ for these well-defined subsets of T-ALL patients.

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Contributions

MR, JM, GJa, TH, and JC are responsible for conception and design. MR, JM, SD, ZK, and GJe provided 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. MR, JM, SD, ZK, GJa, SZ, GK, TH, and JC analyzed and interpreted data. MR wrote the original draft. 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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