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Published in:
Proteomics - Clinical Applications

DOI:
10.1002/prca.202100072

Received: 20 August 2021 | Revised: 30 September 2021 | Accepted: 27 October 2021 | Accepted article online: 1 November 2021

DOI: 10.1002/prca.202100072

Proteomics Clinical Applications

Research Article

Bortezomib is significantly beneficial for de novo pediatric AML patients with low phosphorylation of the NF-κB subunit RelA

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Funding information
NIH COG, Grant/Award Numbers: U10 CA98543, U10 CA98413, U10 CA180886, U24 CA196173, U10 CA180899; NCI, Grant/Award Number: R01-CA164024

Abstract

Purpose: The addition of the proteasome inhibitor (PI) bortezomib to standard chemotherapy (ADE: cytarabine [Ara-C], daunorubicin, and etoposide) did not improve overall outcome of pediatric AML patients in the Children's Oncology Group AAML1031 phase 3 randomized clinical trial (AAML1031) . Bortezomib prevents protein degradation, including RelA via the intracellular NF-kB pathway. In this study, we hypothesized that subgroups of pediatric AML patients benefitting from standard therapy plus bortezomib (ADEB) could be identified based on pre-treatment RelA expression and phosphorylation status.

Experimental design: RelA-total and phosphorylation at serine 536 (RelA-pSer^{536}) were measured in 483 patient samples using reverse phase protein array technology.

Results: In ADEB-treated patients, low-RelA-pSer^{536} was favorably prognostic when compared to high-RelA-pSer^{536} (3-yr overall survival (OS): 81% vs. 68%, $p = 0.032$; relapse risk (RR): 30% vs. 49%, $p = 0.004$). Among low-RelA-pSer^{536} patients, RR significantly decreased with ADEB compared to ADE (RR: 30% vs. 44%, $p = 0.035$). Correlation between RelA-pSer^{536} and 295 other assayed proteins identified a strong correlation with HSF1-pSer^{326}, another protein previously identified as modifying ADEB response. The combination of low-RelA-pSer^{536} and low-HSF1-pSer^{326} was a significant predictor of ADEB response (3-yr OS: 86% vs. 67%, $p = 0.013$).

Conclusion and clinical relevance: Bortezomib may improve clinical outcome in a subgroup of AML patients identified by low-RelA-pSer^{536} and low-HSF1-pSer^{326}.

KEYWORDS
B ortezomib, leukemia, pediatric, proteomics, RPPA

1 | INTRODUCTION

Nuclear factor kappa-B (NF-κB) is a protein complex formed by combinations of the five proteins in the Rel family: RelA (p65), RelB, c-Rel, p50 (NFκ-B1), and p52 (NF-κB2). The subunits RelA, p50, and c-Rel form heterodimers and are held inactive in the cytosol when sequestered by the "inhibitor of kappa B" (IκB) protein. Activation of NF-κB via the canonical pathway is stimulated by multiple pro-inflammatory stimuli or cytokines (e.g., tumor necrosis factor α) that leads to phosphorylation of IκB at serine 32 or 36 by IκB kinase (IKK). Phosphorylation of IκB results in its ubiquitination and proteasomal degradation, releasing the NF-κB complexes from IκB. Additional activation of NF-κB is achieved through phosphorylation of the core component RelA at serine 536 (RelA-pSer^{536}), as well as through other post-translational modifications via IKK [1–3], allowing the untethered complex (RelA/p50) to translocate to the nucleus [4,5]. Here, NF-κB acts as a transcription factor that binds to the κB enhancer motif on DNA. In several cancers, NF-κB is constitutively active as a result of chromosomal translocation amplifications and mutations encoding NF-κB and IκB or IKK proteins [4]. Overexpression of NF-κB target genes (e.g., CREB, c-JUN, GSK3, AP-1) [2,6] is a key survival factor in cancer and plays a key role in cell proliferation, apoptosis [5] and programmed cell death [7]. In AML, NF-κB protein binding is increased in nuclear extracts of leukemic CD34+, but not in those of normal CD34+ cells, suggesting increased NF-κB activation [8].

Survival of pediatric AML remains guarded with 5-year overall survival (OS) rates of around 70% in high-income countries. Although survival exceeds that of solid tumors in pediatrics, there are significant long-term sequelae including heart dysfunction, decreased fertility, and second malignancies [9]. Better therapies are needed to increase survival, reduce relapse, and to reduce long-term side effects [10]. Recently, the Children's Oncology Group (COG) evaluated the efficacy of adding proteasomal inhibition (PI) to standard chemotherapy (ADE: cytarabine (Ara-C), daunorubicin, etoposide) by adding bortezomib (ADEB) in a randomized phase 3 clinical trial for newly diagnosed pediatric AML patients (AAML1031, NCT01371981) [11]. While the study showed no improvement in OS or event-free survival (EFS) across the entire cohort [12], we however, previously showed that ADEB was beneficial in subgroups of patients with decreased phosphorylation of heat shock factor 1 at serine 326 (HSF1-pSer^{326}) [11], as well as in patients demonstrating upregulation of histone modifying enzymes with more transposase-accessible chromatin (submitted).

One of the mechanisms thought responsible for the efficacy of PIs is their antiproliferative and proapoptotic property via manipulation of NF-κB [13]. Initially, it was thought that PIs cause NF-κB inhibition by preventing proteasomal degradation of IκB [5]. However, some studies showed that bortezomib also induced NF-κB activity via phosphorylation of IKK, causing a release of RelA/p50 via increased IκB degradation [14,15]. This indicates that the relationship between NF-κB (in)activity and bortezomib is complex. Given this interaction between NF-κB and bortezomib, we questioned if protein expression of total RelA (RelA-total) or activated RelA (RelA-pSer^{536}) was prognostic of clinical response to ADEB, and if we could identify an additional subgroup of pediatric AML patients that would benefit from ADEB.

2 | METHODS

2.1 | Pediatric AML patient samples

Peripheral blood samples were obtained from 483 de novo pediatric AML patients that participated in the COG AAML1031 (NCT01371981) phase 3 clinical trial, and 30 CD34+ bone marrow samples obtained from healthy donors; of these 20 were pediatric and 10 were adults CD34+ samples. Samples from adults were included to allow future comparison to adult AML samples which were published previously [16]. Samples were acquired during routine diagnostic assessments prior to, 10 h and 24 h after the initiation of systemic chemotherapy, and were collected between July 2011 and February 2017. Written informed consent was obtained in accordance with local IRB review boards and the *Declaration of Helsinki*.

Outcome data was available for 410 of the 483 patients enrolled on the AAML1031 study. One hundred and sixty-four patients received standard ADE induction therapy, 210 patients received ADEB, and 36 patients with known FLT3-ITD mutations received ADE plus sorafenib (ADES). Because we were particularly interested in the association between RelA-total and RelA-pSer^{536} protein expression in relation to treatment with ADE plus bortezomib (ADEB), and since outcome after ADES and ADE did not differ [12], patients treated with ADE and ADES were combined in our proteomic analysis (n = 200). Three hundred forty-eight (85%) patients achieved complete remission by the end of the second course, 31 (8%) patients were refractory or died (failed therapy), 156 (45%) patients relapsed after remission, and 286 were still alive at the end of their follow-up (70%). Survival analysis was performed as published previously [11]. Patients had mutation data available for *CEBPA*, *FLT3-ITD*, *c-KIT*, and *NPM1*.

2.2 | RPPA methodology

The methodology and validation of the RPPA technique have been described elsewhere [17–21]. Briefly, fresh samples were obtained

from patients at local sites and were shipped to the central processing lab at Baylor College of Medicine by overnight courier. Mononuclear cells were isolated from peripheral blood by centrifugation using lymphocyte separation solution (Sigma) and enriched for leukemic cells by CD3/CD19 depletion (Miltenyi Biotech, Cologne, Germany). Protein preparations were normalized to a concentration of 1×10^7 cells/ml and printed in five serial dilutions onto slides along with normalization and expression controls. Slides were probed with 301 antibodies listed in **Table S1**, including a primary validated antibody against total RelA (Cell Signaling, Danvers, MA, Cat. #3034) and RelA-pSer^{536} (Cell Signaling, Cat. #3033). Five antibodies were excluded for technical reasons yielding a final of 296 antibodies used for analysis [11]. Stained slides were analyzed using Microvigene Software (version 3.0, Vigene Tech, Carlisle, MA). *SuperCurve* algorithms were used to generate a single concentration value from the five serial dilutions [22]. Loading control [23] and topographical normalization [24] procedures were performed to account for protein concentration and background antibody staining variations on each array.

2.3 | Transcriptome sequencing data

Additional mutation profiles were obtained from transcriptome sequencing data for 390 of the 483 patients (81%) and generated elsewhere [25]. Mutations that were present in ≥10 patients were *KRAS* ($n = 29$), *NRAS* ($n = 99$), *GATA2* ($n = 12$), *PTPN11* ($n = 28$), *MYH11* ($n = 17$), and *IDH1* or *IDH2* ($n = 14$).

2.4 | Pathway analysis

STRING software (String version 11.0; http://string-db.org) was used to determine protein associations [26].

2.5 | Statistical analysis

Data were frozen as of June 30, 2019 for outcome analyses. Estimates of OS and EFS were calculated using the Kaplan–Meier method. OS and EFS were defined as time from study entry until death or until relapse, secondary malignancy, or death, respectively. RR was calculated using methods of competing events and was defined as the time from the end of two courses of induction (for patients in complete remission) to relapse, where deaths without a relapse were considered competing events. The significance of predictor variables was tested with the log-rank statistic for OS, EFS, and with Gray's statistic for RR. Cox proportional hazard models were used to estimate hazard ratios for univariable and multivariable analyses of OS and EFS. Competing risk regression models were used for analyses of RR. Outcome by treatment arm was based on intention-to treat analysis. Correlation between the patient cohorts and categorical clinical variables were compared using Pearson's Chi-square test, and for continuous variables using the Kruskal-Wallis test. Pearson's correlation analyses were performed to correlate RelA-pSer^{536} protein expression levels with expression of the other 295 proteins. *p*-values were adjusted using false discovery rate correction. All statistical analyses were performed in R Version 1.3.959 (2009-2020, RStudio, Inc., Boston, MA) or SAS version 9.4 (SAS Institute, Inc., Cary, NC).

Clinical Relevance

Survival of pediatric acute myeloid leukemia (AML) is guarded with an overall survival of 70%. Addition of the proteasome inhibitor (PI) bortezomib to standard therapy did not improve outcome overall. In this study, we identified a subgroup of patients with low-RelA-pSer536 that benefitted from additional bortezomib treatment based on proteomics. This finding was even stronger in combination with low-HSF1-pSer326. We hypothesize that if we can identify patients prior to treatment, and treat these with additional therapy, this will result in higher survival rates of pediatric AML. Moreover, the use of a small-molecule RelA inhibitor or inhibitor of an upstream kinase of the NF-κB/RelA pathway could be a potential approach to sensitize patients with high levels of RelA-pSer536 to additional bortezomib. This requires quick and accurate measurement of RelA-pSer536 protein expression. Further research is needed to validate our hypothesis.

3 | RESULTS

3.1 | RelA-total and RelA-pSer^{536} protein expression across newly diagnosed pediatric AML patients

Relative RelA-total and RelA-pSer^{536} protein expression levels were measured in bulk leukemia blasts from 483 pediatric AML patients, as well as in 30 CD34+ samples from healthy controls. All samples used for outcome analysis were collected at time of diagnosis, prior to exposure to systemic chemotherapy. RelA-total was relatively homogeneously expressed, with individual normalized RelA-total expression ranging from −1.84 to +0.94 log2. Overall, RelA-total was not different in pediatric AML compared to normal CD34+ cells (Figure S1A, $p = 0.63$), and only 2% of patients had RelA-total levels significantly higher than normal CD34+ (95% CI normal CD34+ [−0.50; +0.66 log2]). RelA-pSer^{536}, in contrast, had significantly lower expression relative to normal CD34+ cells ($p < 0.001$, Figure S1B), and had a larger expression range (−2.6 to +2.0 log2). Eighty-one percent of the samples had RelA-pSer^{536} expression significantly lower and 6% had expression significantly higher than normal CD34+ (95% CI normal CD34+ [−0.49; +0.49 log2]). RelA-total roughly correlated with RelA-pSer^{536}, with the most variability in phosphorylation

TABLE 1 AML patient characteristics ($n = 483$)

		All cases	Low-RelA-total	High-RelA-total	*p*	Low-RelA-pSer^{536}	High-RelA-pSer^{536}	*p*
Number		100%	50%	50%		50%	50%	
Gender ($n = 481$)	Female	50%	50%	50%	0.49	51%	49%	0.62
Age (years old at study entry)	≤1	12%	10%	14%	0.25	12%	12%	0.13
	2–10	33%	35%	31%		37%	29%	
	≥11	55%	56%	55%		51%	60%	
White blood cell count (at study entry)	>100,000	24%	21%	27%	0.19	27%	20%	0.09
CNS ($n = 480$)	Positive	39%	36%	43%	0.14	40%	38%	0.64
Ethnicity ($n = 480$)	Hispanic	19%	21%	18%	0.55	18%	21%	0.46
Race	Black	12%	11%	13%	0.71	13%	10%	0.53
AAML1031 risk group† ($n = 469$)	High risk	29%	29%	28%	0.99	30%	27%	0.63
Complete remission at end of induction II	Yes	85%	87%	83%	0.30	81%	88%	0.07

†AAML1031 protocol risk group definition: Low risk: inv(16)/t(16;16) or t(8;21), or NPM1 or CEBPα mutation; High risk: FLT3/ITD+ with high allelic ratio ≥ 0.4, or monosomy 5/del5q or 7, without low-risk features; Risk status unknown for 10/410.

occurring in samples with the highest RelA-total (Figure S2A, $r = 0.16$, $p < 0.001$).

RelA-total and RelA-pSer^{536} were also measured in different myeloid cell populations with varying degrees of stem cell characteristics. Compared to leukemic bulk and CD3/CD19 depleted mononuclear cells, RelA-total was lower in CD34+, CD34+CD38+ and stem cell enriched CD34+CD38- populations ($p < 0.001$, $p = 0.006$, and $p = 0.019$, respectively, Figure S1C). RelA-pSer^{536} expression was lowest in CD34+ ($p < 0.001$) and highest in CD34-CD38- ($p < 0.001$) and CD34-CD38+ ($p < 0.001$) compared with the CD3-CD19- leukemic bulk cells (Figure S1D). RelA-pSer^{536} median expression trended slightly higher in cells with markers of leukemia stem cells (LSC) (LSC-like; CD34+CD38-) compared to bulk cells ($n = 33$, $p = 0.053$). Yet, a significant difference between the variances of RelA-pSer^{536} was observed in LSC versus bulk cells (Bartlett test of homogeneity of variances, $p = 0.002$). This suggests that RelA-pSer^{536} levels tend to be higher in LSC-enriched AML blasts compared to bulk and CD3-/CD19- cells, although median expressions are not significantly different.

In addition, RelA mRNA transcriptome levels were available for 390 of the 483 AML patients. There was no significant correlation between RelA mRNA and RPPA protein levels for RelA-total (Pearson's coefficient $r = 0.057$, $p = 0.27$) or between RelA mRNA and RelA-pSer^{536} ($r = 0.027$, $p = 0.6$) (Figure S2B and C).

3.2 | Correlation between RelA, patient characteristics and disease features

To associate RelA-total and RelA-pSer^{536} expression levels with patient characteristics (Table 1) and molecular AML features (Table 2), patients were divided into two groups based on median RelA expression across the 483 patients of both RelA antibody-targets. While the frequency of most variables was not different between the two cohorts, low-RelA-total-patients were more frequently associated with t(8;21) translocation and *c-Kit* mutation, and less frequently with *CEBPA* and *GATA2* mutation. Among all the clinical features that were compared, patients with low-RelA-pSer^{536} only differed from high-RelA-pSer^{536} patients in frequency of *IDH1/2* (6% vs. 1%, $p = 0.02$).

3.3 | Low-RelA-pSer^{536} correlates with favorable outcome in patients treated with ADEB treatment

To investigate the effect of RelA-total and RelA-pSer^{536} on outcome, survival analysis was performed for 410 of the 483 patients with available outcome data. OS, EFS, and RR were calculated between low- and high-RelA-total, and low- and high-RelA-pSer^{536}. No prognostic effects were observed for RelA-total across the entire cohort, or when stratified by ADE versus ADEB therapy (Figure S3). However, when we performed the same analysis in RelA-pSer^{536}, low-RelA-pSer^{536} was significantly favorable in ADEB treated patients (Figure 1A, solid lines). In ADEB-treated patients, 3-yr-OS was 81% in the low-RelA-pSer^{536} patients compared with 68% in high-RelA-pSer^{536} ($p = 0.032$), and 3-yr post-induction RR was 30% in low-RelA-pSer^{536} patients versus 49% in high-RelA-pSer^{536} patients ($p = 0.004$, Figure 1C). A similar trend was observed when RelA-pSer^{536} was split into thirds. Low-RelA-pSer^{536} patients treated with ADEB also trended toward better 3-yrs EFS, with 60% 3-yrs EFS vs. 47% in high-RelA-pSer^{536} ($p = 0.058$, Figure 1B). Differences in outcome between low- and high-RelA-pSer^{536} were not observed in patients treated with ADE ($p = 0.985$, Figure 1B).

TABLE 2 Pediatric AML molecular features ($n = 483$)

		All cases	Low-RelA-total	High-RelA-total	*p*	Low-RelA-pSer^{536}	High-RelA-pSer^{536}	*p*
Number		100%	50%	50%		50%	50%	
Cytogenetics ($n = 476$)	t(8;21)	16%	22%	10%	**0.001**	16%	16%	0.93
	inv16	14%	13%	14%	0.89	16%	11%	0.16
	Normal karyotype	28%	25%	31%	0.22	28%	27%	0.88
	t(9;11)(p22;q23)/11q23	18%	14%	21%	0.06	14%	22%	0.05
	−5, −7, or +8	9%	8%	10%	0.75	10%	8%	0.44
	Other	15%	17%	14%	0.45	14%	16%	0.87
NPM1 mutation	Mutant	10%	12%	9%	0.30	7%	13%	0.05
CEBPA mutation	Mutant	9%	5%	13%	**0.001**	12%	6%	0.06
FLT3-ITD mutation	Mutant	22%	21%	23%	0.64	22%	21%	0.84
High-allelic FLT3-ITD ratio	Yes (≥ 0.4)	74%	79%	77%	0.58	77%	71%	0.57
c-Kit mutation (exon 8) ($n = 399$)	Mutant	4%	6%	2%	0.19	5%	3%	0.47
c-Kit mutation (exon 17) ($n = 399$)	Mutant	8%	11%	5%	0.09	7%	9%	0.80
c-Kit mutation (exon 8 or 17) ($n = 399$)	Mutant	12%	16%	8%	0.026	12%	12%	1.00
KRAS mutation ($n = 390$)	Mutant	7%	6%	9%	0.44	8%	7%	1.00
NRAS mutation ($n = 390$)	Mutant	25%	26%	25%	0.82	26%	25%	0.91
KRAS or NRAS mutation ($n = 390$)	Mutant	31%	31%	30%	1.00	30%	31%	1.00
PTPN11 mutation($n = 390$)	Mutant	7%	9%	6%	0.33	6%	9%	0.30
MYH11 mutation ($n = 390$)	Mutant	4%	5%	4%	0.62	6%	3%	0.34
GATA2 mutation ($n = 390$)	Mutant	3%	1%	6%	**0.008**	4%	3%	0.80
IDH1 or 2 ($n = 390$)	Mutant	4%	3%	5%	0.41	6%	1%	**0.02**

*NA/ unknown values not considered in *p*-value calculations and are excluded from the results.

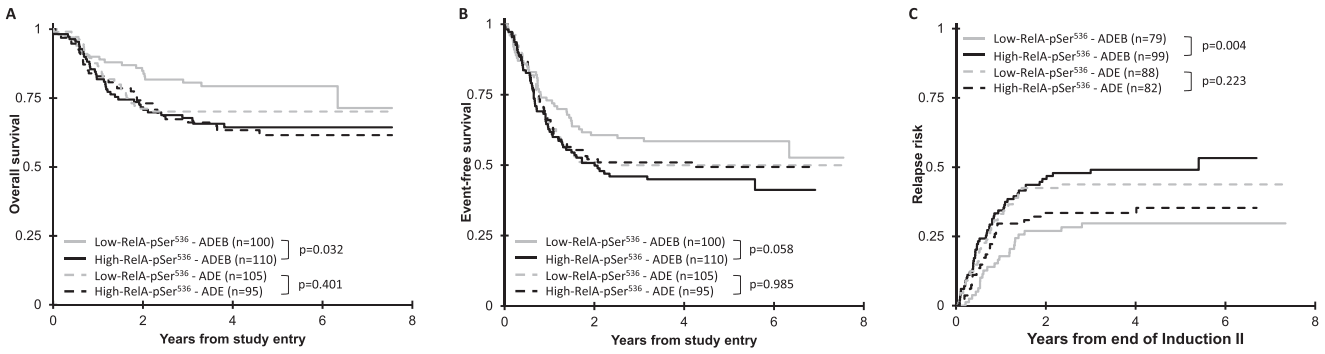

FIGURE 1 Kaplan-Meier survival analysis for low and high-RelA-pSer^{536}. (A) OS, (B) EFS, and (C) RR for low-RelA-pSer^{536} patients treated with ADE or ADES (gray dashed line) or ADEB (gray solid line), versus high-RelA-Ser^{536} patients treated with ADE or ADES (black dashed line) or ADEB (black solid line)

Comparison of outcome between the treatment arms in low- and high-RelA-pSer^{536} patients separately, showed a significant decreased in RR after ADEB in low-RelA-pSer^{536} compared with ADE (30% vs. 44%, $p = 0.035$). OS and EFS also trended to be better after ADEB, but differences were not significant (70% vs. 81%, $p = 0.159$; 50% vs. 60%, $p = 0.257$) (Figure 1C, gray lines). In high-RelA-pSer^{536} patients, RR tended to show the reverse, with better outcome after ADE versus ADEB (RR, $p = 0.051$, Figure 1C, black lines). When restricted to

low-risk AAML1031 patients only [12], survival analysis again identified low-RelA-pSer^{536} as favorable prognostic indicator in ADEB-treated patients (OS, $p = 0.045$, EFS $p = 0.057$, RR $p = 0.010$) (Figure S4). Multivariate analysis revealed high-RelA-pSer^{536} as an independently unfavorable prognostic variable for RR with ADEB treatment (Table S2).

3.4 | RelA-pSer^{536} phosphorylation increases following systemic chemotherapy

To gain insight into the effect of bortezomib on RelA expression and activation state in AML, we analyzed baseline RelA-total and RelA-pSer^{536} expression, and compared this to expression in samples collected 10 and 24 h after the initiation of systemic treatment. Although, RelA-total did not show any change after ADE or ADEB treatment over 24 h (Figure S5), we observed that RelA-pSer^{536} expression was increased 24 h after exposure to both ADE and ADEB compared to pre-treatment (Figure S6**A**). Because we had seen that outcome of ADEB-treated patients significantly improved in low-RelA-pSer^{536} patients but not in high-RelA-pSer536, we evaluated changes in expression after chemotherapy in low-RelA-pSer^{536} and high-RelA-pSer^{536} patients. This showed an increase in RelA-pSer^{536} after 24 h in low-RelA-pSer^{536} patients independent of their received treatment ($p = 5.2e\text{-}06$, $p = 1.3e\text{-}07$, respectively, Figure S6**B**), but not in high-RelA-pSer^{536} patients ($p = 0.69$, ADEB; $p = 0.42$, Figure S6**C**). Expression in patients with low-pretreatment RelA-pSer^{536} did not reach the pre-treatment levels of the high-RelA-pSer^{536} patients.

3.5 | RelA-pSer^{536} positively correlates with HSF1-pSer^{326}

RelA-pSer^{356} expression was correlated with 295 proteins on the same array. Correlations were performed by calculating Pearson's correlation coefficient. The strongest correlation to RelA-pSer536 was MAPK14-pThr180_Tyr182 ($r = 0.65$, $p < 0.001$), a kinase known to facilitate activation of transcription factors, including RelA in the NF-κB complex.[27,28] Twenty-four proteins were identified as being positively correlated with RelA-pSer^{536}, and four proteins (*PRKCB*, *GRP78*, *MYH11*, *ELK1*.pSer^{383}) were negatively correlated ($r > 0.25$, Bonferroni adjusted $p < 0.05$) (**Figure 2A**). None of the proteins was associated with cell death. All but two positively correlated proteins were phosphorylated.

Interestingly, low expression of the protein HSF1-pSer^{326}, previously found to be associated with the beneficial effect of ADEB in pediatric AML, was among the strongest correlated proteins with low RelA-pSer^{536} ($r = 0.59$, $p < 0.001$) [11]. Furthermore, twenty-four of the 28 proteins that correlated with RelA-pSer^{536} also significantly correlated with HSF1-pSer^{326} (Figure S7). Although a search in the STRING database revealed no previously known relationship between these proteins, network analysis connected RelA and HSF1 via MAPK1, both directly and through several other proteins (**Figure 2B**). To see if this same relationship exists in other hematological malignancies, we performed the identical analysis in 361 T-cell acute lymphoblastic leukemia patient samples ($n = 268$ pediatric, $n = 93$ adult). Again, HSF1-pSer^{326} was found to be the most significantly correlated protein with RelA-pSer^{536} ($r = 0.56$, $p < 0.001$). Also, out of the 22 positively correlated proteins, 14 proteins contained post-translationally modified protein sites (Figure S8).

3.6 | The combination of low-RelA-pSer^{536} and low-HSF1-pSer^{326} augments the beneficial effect of bortezomib

Because RelA-pSer^{536} and HSF1-pSer^{326} were correlated, and because both proteins were identified as individually prognostic in pediatric AML patients treated with ADEB, we hypothesized that the combination of RelA-pSer^{536} and HSF1-pSer^{326} might have increased survival or lower relapse rates. Therefore, patients were clustered into four groups based on the combination of the expression of these two proteins; low-RelApSer^{536}-low-HSF1-pSer^{326} ($n = 183/483$, 38%), low-RelA-pSer^{536}-high-HSF1-pSer^{326} ($n = 59$, 12%), high-RelA-pSer^{536}-low-HSF1-pSer^{326} ($n = 59$, 12%), high-RelA-pSer^{536}-high-HSF1-pSer^{326} ($n = 182$, 38%). The unequal distribution of patients among these four groups suggests a strong linkage between RelA-pSer^{536} and HSF1-pSer^{326} expression (Chi-Square test, $p < 0.01$). Patient characteristics based on these four protein groups are shown in Table S3.

Survival analysis, restricted to patients treated with ADEB, showed that the combination of low-RelApSer^{536}-low-HSF1-pSer^{326} had a superior 3-yr OS and EFS compared to the other three groups (**Figure 3**, OS; 86% vs. 67%, $p = 0.001$, EFS: 66% vs. 45%, $p = 0.002$). RR was also significantly lower in patients with the combination of low-RelApSer^{536}-low-HSF1-pSer^{326} compared to the other patient groups (26% vs. 48%, $p = 0.002$). Again, this effect was not seen in ADE-treated patients (Figure S9)

3.7 | The combination of low-RelA-pSer^{536} and low-HSF1-pSer^{326} is an independent prognostic marker in ADEB treatment

To investigate whether the prognostic effect of the combination of RelA-pSer536 and HSF1-pSer326 in ADEB-treated patients was independent of other variables, multivariate analysis was performed. Only variables found to be significantly prognostic in univariate analysis were considered. This analysis showed a significant contribution on outcome for RelA-pSer^{536} with HSF1-pSer^{326}, age at diagnosis and AAML1031 risk groups. Hazard ratio of low-RelA-pSer^{536} with low HSF1-pSer^{326} (low-low) was set as reference (HR = 1), with associated increase HR for the remaining three subsets (low-high, high-low, high-high, **Table 3**).

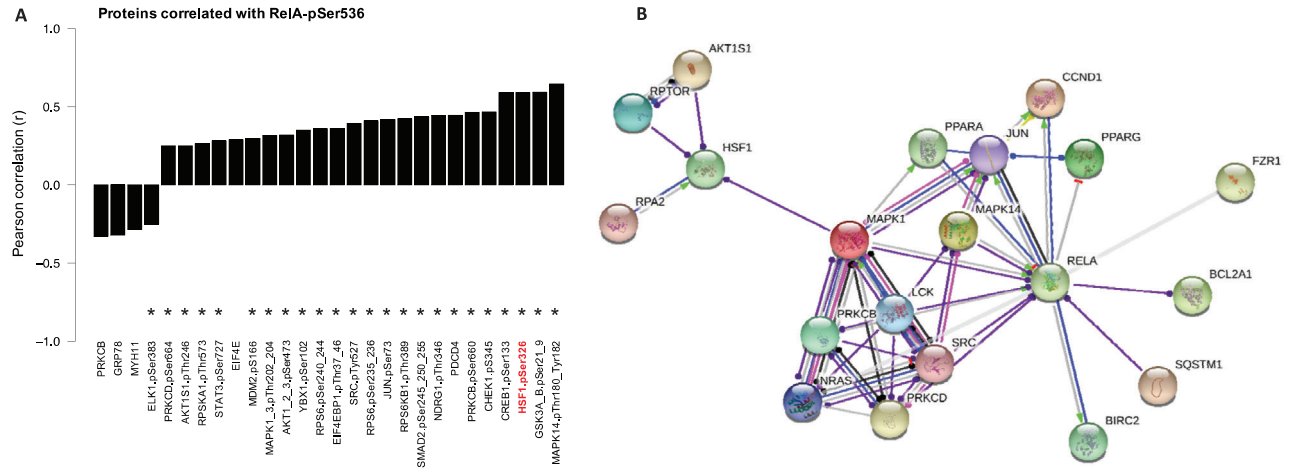

FIGURE 2 Waterfall plot and network analysis of correlated proteins with RelA-pSer^{536}. (A) Waterfall plot showing the 28 significantly correlated proteins with RelA-pSer536 ($r \geq 0.25$ or $r \leq -0.25$). * Denotes antibodies directed against PTM-sites. (B) STRING networks analysis for the 24 proteins that correlated with both RelA-pSer^{536} and HSF1-pSer^{326}. Only interactions with either RELA or HSF1 are shown

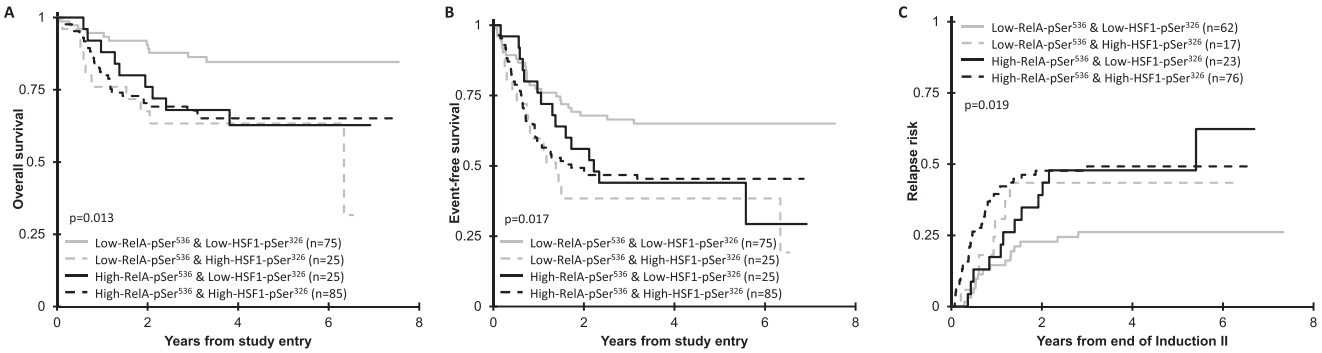

FIGURE 3 Kaplan-Meier survival analysis for RelA-pSer^{536} and HSF1-pSer^{326} combined in ADEB-treated patients. (A) OS, (B) EFS, and (C) RR for patients stratified based on their RelA-pSer^{536} and HSF1-pSer^{326} expression levels. Solid gray: low-RelA-pSer^{536}, low-HSF1-pSer^{326}; dashed gray: low-RelA-pSer^{536}, high-HSF1-pSer^{326}; solid black: high-RelA-pSer^{536}, low-HSF1-pSer^{326}; dashed black: high-RelA-pSer^{536}, high-HSF1-pSer^{326}

TABLE 3 Multivariate analysis in ADEB-treated patients, including the combination of RelA-pSer^{536} and HSF1-pSer^{326}

		OS from study entry				EFS from study entry			RR from end of course 2			
		N	HR	95% CI	*p*	HR	95% CI	*p*	N	HR	95% CI	*p*
RELA-pSer536 & HSF1-pSer326	Low-low	71	1			1			62	1		
	Low-high	25	3.74	1.50 - 9.36	**0.005**	2.46	1.29 - 4.71	**0.007**	17	1.85	0.76 - 4.50	0.177
	High-low	25	2.26	0.88 - 5.79	0.090	1.51	0.78 - 2.90	0.220	23	1.88	0.91 - 3.89	0.089
	High-high	83	2.96	1.38 - 6.32	**0.005**	1.97	1.19 - 3.26	**0.008**	76	2.62	1.44 - 4.76	**0.002**
Age at Dx	0-1 yr.	27	2.22	1.05 - 4.71	**0.038**	2.26	1.23 - 4.13	**0.008**	22	2.02	0.97 - 4.23	0.062
	2-10 yr.	67	1			1			57	1		
	11+ yr.	110	0.72	0.39 - 1.31	0.283	0.77	0.50 - 1.21	0.257	99	0.71	0.42 - 1.22	0.214
Risk group (AAML1031 definition)†	Low	155	1			1			146	1		
	High	49	2.80	1.58 - 4.97	<**0.001**	2.31	1.47 - 3.64	<**0.001**	32	1.53	0.82 - 2.85	0.186

4 | DISCUSION

In this study we showed for the first time that low-RelA-pSer^{536} was associated with better outcome compared to high-RelA-pSer^{536} in pediatric AML patients treated with ADEB, and that patients with low-RelA-pSer^{536} did better after treatment with ADEB versus ADE alone, whereas high-RelA-pSer^{536} did worse with ADEB. We also showed that RelA-pSer^{536}, but not RelA-total, increased following chemotherapy in the low-RelA-pSer^{536} patients but not in high-RelA-pSer^{536} patients. Previously we demonstrated that subgroups of pediatric AML patients benefitted from ADEB, including those with low-HSF1-pSer^{326}.

In addition to low-RelA-pSer^{536}, we also showed that the combination of low-RelA-pSer^{536} and low-HSF1-pSer^{326} improved prognosis compared with either protein alone. We suggest that the combination of these two proteins could be used to a priori identify a group of AML patients (38% of patients in this study) that benefit from ADEB. Of note, while the combination with low/high expression of histone modifying enzymes did not affect patient prognosis with both low-RelA-pSer^{536} and low-HSF1-pSer^{326} expression, HME stratification could potentially identify another small group of low-RelA-pSer^{536} and high-HSF1-pSer^{326} expression with a favorable prognosis after ADEB, while the low-RelA-pSer^{536} and high-HSF1-pSer^{326} following ADE did not (OS; $p = 0.025$, EFS; $p = 0.007$, RR; $p = 0.085$, **Figure S10**).

Studies have shown that proteasome inhibition has two separate effects on the NF-κB. Bortezomib and other PIs can either block proteasomal degradation of IkB, resulting in decreased NF-kB activation, or phosphorylate the NF-κB regulator IKK, which leads to increased NF-κB activation [5,27]. We evaluated RelA expression and activation (i.e., phosphorylation) across pediatric AML subtypes and measured RelA expression across a variety of AML cell types. Our data showed that only 2% of bulk leukemic cells had RelA-total significantly higher than in normal CD34+ and only 9% of bulk leukemia had higher RelA-pSer^{536} than in normal expression of RelA-pSer^{536}. Previous work has shown that RelA is often up-regulated in AML, but those studies were conducted in tumor cell lines and may not accurately reflect the biology in primary cells, or measured RelA-activity in the leukemic stem cell population, which are known to have higher NF-κB expression levels compared to bulk cells. Despite small numbers ($n = 33$), our data showed a similar trend toward higher expression of RelA-pSer^{536} in LSC-enriched AML cells, which supports the finding that NF-κB expression/activation is likely higher in leukemia initiating stem cells [8].

Secondly, when we correlated RelA expression with outcome using multivariate analysis, we identified RelA-pSer^{536} as a prognostic indicator in patients treated with ADEB. Patients with low-RelA-pSer^{536} expression had a significantly better survival compared to those with the high RelA-pSer^{536} levels (+13% OS, −19% RR at 3-yr). We think that as bortezomib primarily inhibits NF-kB activation and in turn induces cell death, low-RelA-pSer^{536} can enhance the effect of low NF-κB activity.

If we are able to identify this subset of low-RelA-pSer^{536} patients prior to treatment, those could potentially be treated with ADEB, resulting in higher survival rates. However, this requires quick measurement of RelA-pSer^{536} expression at the time of diagnosis to accurately distinguish high from low-RelA-pSer^{536} patients (e.g., by using an enzyme-linked immune sorbent assay or immunohistochemistry). In addition, the use of a small molecule inhibitor to directly inhibit RelA or an upstream activating kinase of the NF-κB/RelA pathway, would be another approach to sensitize high-RelA-pSer^{536} patients to ADEB.. For instance, in chronic inflammatory diseases, where a disproportional activation of RelA is often part of the underlying inflammatory pathology, there has already been tremendous focus on manipulating RelA [28,29]. A challenge in creating RelA as a therapeutic target remains proper intracellular delivery and selective targeting without significant off-target effects. A promising drug that recently entered a phase I clinical trial for hematological malignancies, including AML, myelodysplastic syndrome and non-Hodgkin lymphoma, is the interleukin-1 receptor associated kinase (IRAK) inhibitor CA-4948. IRAK inhibitors manipulate NF-κB mediated transcription via inhibition of IRAK, which is an upstream activating kinase of the IKK complex [29].

As in various hematological malignancies, treatment with PIs was shown to induce activation of NF-kB in a time- and dose-dependent manner [30–32], we compared RelA-total and RelA-pSer^{536} expression pre-treatment to expression 10 and 24 h post-treatment exposure. Whereas RelA-total levels did not change overtime, RelA-pSer^{536} significantly increased 24 h following treatment regardless of treatment regimen. We hypothesize that chemotherapy, regardless of the addition of bortezomib, induces RelA phosphorylation as an attempt to respond to stress caused by the chemotherapy. In addition, phosphorylation levels of low-RelA-pSer^{536} patients significantly increased after both ADE and ADEB treatment, but this effect did not occur in the high-RelA-pSer^{536} patients. Low-RelA-pSer^{536} patients might be more dependent on this stress-response than high-RelA-pSer^{536} for survival, which is speculatively blocked by the PI addition, leading to more cell stress and cell death resulting in better clinical outcome. Although increased, the change after 24 h in low-RelA-pSer^{536} cells was not strong enough to reach the baseline levels seen in high-RelA-pSer^{536} patients. A schematic summary of our hypothesis is shown in Figure 4.

Finally, we found a strong correlation between RelA-pSer^{536} and HSF1-pSer^{326}, which was strengthened by a similarly strong association in a cohort of 358 T-cell acute lymphoblastic leukemia patients, increasing the probability that there is a real, but previously unrecognized, relationship between these proteins. Since low levels of both protein modifications were favorably prognostic in ADEB-treated patients, the combination of low-RelA-pSer^{536} and low-HSF1-pSer^{326} appears to increase our ability to predict which patients are likely to respond to PI-containing chemotherapy. The favorable prognostic effect of low expression of RelA-pSer536 and HSF1-pSer326 was abolished when the expression of either protein was high. We hypothesize that as both proteins become active in response to cell stress,

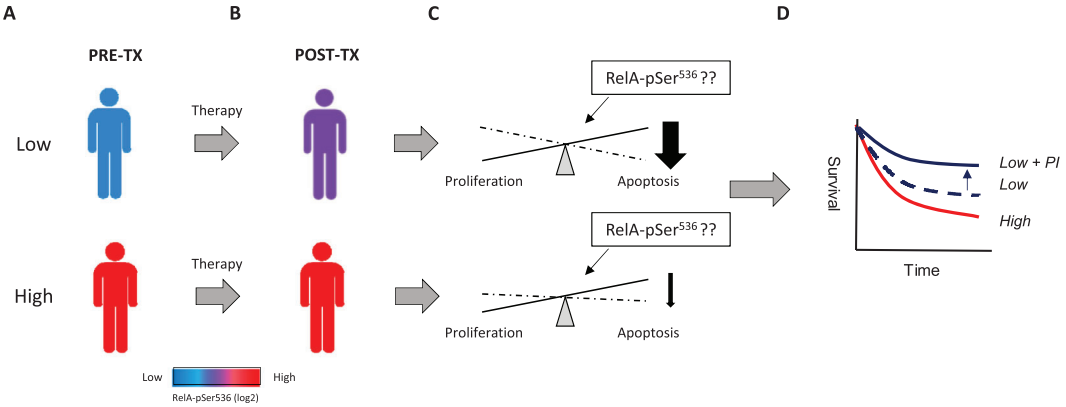

FIGURE 4 Schematic summary of RelA-pSer536 dependence in bortezomib treatment. (A) Patients were split based on pre-treatment RelA-pSer536 expression into low (blue) and high (red). (B) Expression increases in low-RelA-pSer536 patients after treatment with chemotherapy (purple), but does not reach the same levels as found in the high-RelA-pSer536. In high-RelA-pSer536 patients, no increase in RelA-pSer536 was seen. (C) Solid line represents baseline state of the leukemia cells with a tendency toward cell proliferation. After chemotherapy, this balances over to more apoptosis. We hypothesize that RelA-pSer536 plays a role in stress response caused by chemotherapy which is speculatively blocked by PI therapy, and that low-RelA-pSer536 patients are more dependent on this response. Size of the black arrow represents the increase in apoptosis. (D) Increased cell stress result in cell death and cell death eventually leads to patient survival. Survival of low-RelA-pS536 increases after treatment with ADEB (solid line) versus ADE (dashed line)

the combination of low-RelA/HSF1 represents the intrinsically most "stressless" state. This suggests that these low-low AML cells may be more sensitive to increased stress caused by PI inhibition. The buildup of misfolded proteins in AML cells with low RelA/HSF1 may prevent adaption to homeostasis disruption, resulting in cell death. This study suggests a functional HSF1-RelA axis in AML in response to proteasome inhibition. We were able to link RelA to HSF1 via MAPK1, directly or via several other proteins (Figure 2). The observation that a better prognosis in pediatric AML after ADEB in patients with both HSF1 and RelA provides further evidence that a RelA-NF-kB pathway may be relevant to chemotherapy containing PI. Rao et al. found that knockdown of HSF1 results in inhibition of the NF-κB pathway [33], but more research is needed to verify the existence of a RelA-HSF1 axis and to confirm its role in AML PI sensitivity.

In conclusion, in this study we have identified low-RelA-pSer^{536} as favorable prognostic factor in ADEB treated pediatric AML patients. This finding was even stronger in combination with HSF1-pSer^{326}. As about one third of the patients expressed low-RelA-pSer^{536} and low-HSF1-pSer^{32}, we hypothesize that a priori identification and treatment with ADEB of these patients may result in a significant improvement of OS in pediatric AML.

ACKNOWLEDGMENTS

TMH, RBG, ASG, RA, EAK, and RAA were funded by the NIH COG Grants U10 CA98543, U10 CA98413, U10 CA180886, U24 CA196173, and U10 CA180899. TMH was funded by the NCI R01-CA164024, a grant from Hope on Wheels, Hyundaii Foundation, the St. Baldrick's Foundation, and a grant from Takeda Pharmaceuticals. ADvD and FWH were funded by the Junior Scientific Masterclass (Groningen, the Netherlands).

CONFLICT OF INTERESTS

TMH receives research funding from Takeda Pharmaceuticals.

DATA AVAILABILITY STATEMENT

RPPA data used for the analysis was deposited at https://www.leukemiaatlas.org/).

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SUPPORTING INFORMATION

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How to cite this article: van Dijk, A. D., Hoff, F. W., Qiu, Y., Gerbing, R. B., Gamis, A. S., Aplenc, R., Kolb, E. A., Alonzo, T. A., Meshinchi, S., Jenkins, G., de Bont, E. S. J. M., Kornblau, S. M., & Horton, T. M. (2021). Bortezomib is significantly beneficial for *de novo* pediatric AML patients with low phosphorylation of the NF-κB subunit RelA. *Proteomics – Clinical Applications*, e2100072. https://doi.org/10.1002/prca.202100072