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TUMOR MARKERS AND SIGNATURES

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Serum levels of soluble B and T lymphocyte attenuator predict overall survival in patients undergoing immune checkpoint inhibitor therapy for solid malignancies

Joao Gorgulho[1,2] | Christoph Roderburg[3] | Felix Heymann[4] | Maximilian Schulze-Hagen[5] | Fabian Beier[6] | Mihael Vucur[3] | Jakob N. Kather[7] | Narmin Ghaffari Laleh[7] | Frank Tacke[4] | Tim H. Brümmendorf[6] | Tom Luedde[3] | Sven H. Loosen[3]

[1]Division of Gastroenterology, Hepatology and Hepatobiliary Oncology, University Hospital RWTH Aachen, Aachen, Germany

[2]Department of Oncology, Hematology and Bone Marrow Transplantation with Section of Pneumology, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany

[3]Clinic for Gastroenterology, Hepatology and Infectious Diseases, University Hospital Düsseldorf, Medical Faculty of Heinrich Heine University Düsseldorf, Düsseldorf, Germany

[4]Department of Hepatology and Gastroenterology, Charité University Medicine Berlin, Berlin, Germany

[5]Department of Diagnostic and Interventional Radiology, University Hospital RWTH Aachen, Aachen, Germany

[6]Department of Medicine IV, University Hospital RWTH Aachen, Aachen, Germany

[7]Department of Medicine III, University Hospital RWTH Aachen, Aachen, Germany

Correspondence
Tom Luedde, Professor of Medicine, Clinic for Gastroenterology, Hepatology and Infectious Diseases, University Hospital Düsseldorf, Medical Faculty of Heinrich Heine University Düsseldorf, Moorenstraße 5, 40225 Düsseldorf, Germany.
Email: luedde@hhu.de

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Abstract

Therapy with immune checkpoint inhibitors (ICIs) can lead to durable tumor control in patients with various advanced stage malignancies. However, this is not the case for all patients, leading to an ongoing search for biomarkers predicting response and outcome to ICI. The B and T lymphocyte attenuator (BTLA) is an immune checkpoint expressed on immune cells that was shown to modulate therapeutic responses. Here, we evaluate circulating levels of its soluble form, soluble B and T lymphocyte attenuator (sBTLA), as a biomarker for the prediction of treatment response and outcome to ICI therapy. Serum levels of sBTLA were analyzed by multiplex immunoassay in n = 84 patients receiving ICI therapy for solid malignancies and 32 healthy controls. BTLA expression was evaluated on peripheral blood mononuclear cells in a subset of patients (n = 6) using multicolor flow cytometry. Baseline sBTLA serum levels were significantly higher in cancer patients compared to healthy controls. Importantly, circulating sBTLA levels were an

Abbreviations: APC, antigen presenting cell; BTLA, B and T lymphocyte attenuator; CR, complete remission; CTL, cytotoxic T lymphocyte; DC, disease control; HVEM, herpes virus entry mediator; ICI, immune checkpoint inhibitor; IRAE, immune-related adverse effect; LAG-3, lymphocyte activation gene 3; NSCLC, nonsmall cell lung cancer; OS, overall survival; PBMC, peripheral blood mononuclear cell; PD, progressive disease; PR, partial remission; SD, stable disease; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; TIL, tumor-infiltrating lymphocyte; TIM-3, T-cell immunoglobulin and mucin-domain containing protein 3; UICC, Union for International Cancer Control.

Joao Gorgulho and Christoph Roderburg share the first authorship.

Tom Luedde and Sven H. Loosen share the last authorship.

independent prognostic factor for overall survival (OS). As such, patients with initial sBTLA levels above the calculated prognostic cutoff value (311.64 pg/mL) had a median OS of only 138 days compared to 526 for patients with sBTLA levels below this value ($P = .001$). Uni- and multivariate Cox regression analyses confirmed the prognostic role of sBTLA in the context of ICI therapy. Finally, we observed a significant correlation between sBTLA levels and the frequency of CD3 + CD8 + BTLA+ T cells in peripheral blood. Thus, our data suggest that circulating sBTLA could represent a noninvasive biomarker to predict outcome to ICI therapy, helping to select eligible therapy candidates.

KEYWORDS
biomarker, BTLA, checkpoint inhibitors, CTLA-4, immunotherapy, nivolumab, PD-1, PD-L1, pembrolizumab, prognosis

1 | INTRODUCTION

Cancer has remained one of the main causes of death on a global scale. In 2018, an estimated 9.6 million cancer deaths occurred.[1,2] Over the past few decades, therapeutic alternatives for advanced stage cancer have lacked to emerge. The development of recent alternatives in immunotherapy such as immune checkpoint inhibitors (ICIs) has revolutionized the therapy of solid malignancies such as malignant melanoma, achieving 5-year survival rates of up to 50% even in metastatic patients,[3] and nonsmall cell lung cancer (NSCLC).[4] Approved ICIs include, among others, the anti-CTLA-4 antibody ipilimumab,[3] the anti-PD-1 agents nivolumab,[4] and pembrolizumab,[5] as well as the PD-L1 blocking antibodies durvalumab,[6] avelumab[7] or atezolizumab.[8] However, since their approval and implementation in different clinical settings, response rates and toxicities to ICIs have diverged between patients. Thus, there is a need for preselection criteria that can accurately predict whether a patient would respond to and tolerate this novel therapeutic approach.[9]

The B and T lymphocyte attenuator (BTLA) is a coinhibitory receptor expressed on the surface of T cells, B cells, dendritic cells and myeloid cells and possesses an extracellular domain of the immunoglobulin family. This domain binds to the herpes virus entry mediator (HVEM), a protein of the tumor necrosis factor family, which is also widely expressed in a variety of immune cells.[10] Upon activation, BTLA acts identically or even synergistically to PD-1 and CTLA-4, delivering a co-inhibitory signal that leads to T-cell inhibition and anergy, playing an irrefutable role in modulating the immune response against cancers,[11] and possibly in influencing the effectiveness of ICI-based cancer immunotherapy. Its soluble form, sBTLA, has previously been described as a promising biomarker in the landscape of sepsis, inflammation[12] and cancer,[13,14] but its role in the context of ICI has remained obscure. In the present study, we therefore evaluated whether serum concentrations of circulating sBTLA could play a role as a potential biomarker to predict tumor response, tolerance and outcome to ICI therapy in different solid malignancies.

What's new?

While immune checkpoint inhibitors (ICIs) boost survival in some cancer patients, response rates and toxicities vary. Anticipating divergent responses and tolerance to ICIs requires the discovery of novel biomarkers and predictive criteria. This study identifies a predictive association between soluble B and T lymphocyte attenuator (sBTLA), an immune regulatory receptor, and overall survival following ICI therapy in a range of tumor entities. Patients with sBTLA levels below an ideal cut-off value before and during ICI treatment survived significantly longer compared to patients with high sBTLA concentrations. Analyses further show that sBTLA levels correlate with BTLA expression on peripheral cytotoxic lymphocytes.

2 | PATIENTS AND METHODS

2.1 | Study population and composition

We elaborated this observational cohort study with the objective of assessing the impact of serum levels of circulating sBTLA on tumor response, toxicity and outcome in patients with different solid malignancies receiving ICIs. The study population consisted of n = 84 patients who were submitted to ICI therapy at the interdisciplinary cancer outpatient clinic at University Hospital RWTH Aachen for advanced-stage disease (Union for International Cancer Control [UICC] III or IV). The patients were prospectively recruited from August 2017 to September 2019 and enrolled in this study (see Table 1 for patient characteristics). The study protocol was approved by the ethics committee of the University Hospital RWTH Aachen, Germany (EK 206/09) and was conducted in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its

later amendments. Written informed consent was obtained from the patients. Blood samples were collected prior to ICI therapy and during the course of treatment at two different time points (early time point: after one to two cycles of therapy; late time point: after three to five cycles). From these samples, we isolated the serum by centrifugation for 10 minutes at 2000*g* and stored them at −80°C until use. For the isolation of peripheral blood mononuclear cells (PBMCs), we drew blood using the BD Vacutainer CPT System (Cat No. 362782, BD Bioscience), followed manufacturer's instructions for isolation[15] and cryopreserved them with 10% DMSO at −80°C. In addition, a total of n = 32 healthy, cancer-free blood donors were used as a control population for serum analyses. Therapy response assessment was based on clinical and radiological evaluation. Responders were categorized in the group "disease control" (DC, including complete response (CR), partial response (PR) and stable disease (SD)) and nonresponders, who exhibited progressive disease (PD), labeled as "non-DC".

2.2 | Evaluation of sBTLA serum levels

Serum concentrations of sBTLA were analyzed by multiplex immunoassay according to the manufacturers instruction using a Bio-Plex 200 system and Bio-Plex Manager 6.0 software (Immuno-Oncology Checkpoint 14-Plex Human ProcartaPlex Panel 1, #EPX14A-15 803-901, Thermo Fisher Scientific).

2.3 | Evaluation of BTLA surface expression of PBMCs with multicolor flow cytometry

In a subset comprising n = 6 patients, we evaluated the surface expression of certain molecules relevant to the immune response on PBMCs, known to play a part in the antitumoral immunity and the efficacy of ICI therapy.[11,16] To differentially investigate the lymphocyte subpopulations in the peripheral blood, we used multicolor flow cytometry analysis. Since T-cell-mediated cytotoxicity, mainly by cytotoxic T lymphocytes (CTL), expressing CD3 and CD8 at the cell surface, is considered the most important axis in lymphocyte-associated tumor control, we next focused on the CTL response in more detail, checking for cell-bound BTLA on different T-cell populations, taking special interest in CD3 + CD8 + BTLA+ cells. We therefore thawed the priorly isolated PBMCs and stained them for flow cytometry analysis using fluorescently labeled monoclonal antibodies directed toward different markers to discriminate between T-helper cells (CD3 + CD4+) and cytotoxic T cells (CD3 + CD8+), and investigated their surface expression of coinhibitory BTLA (CD3: Clone UCHT1, Fluorophor APC-R700; CD4: Clone SK3, Fluorophor BUV737; CD8: Clone RPA-T8, Fluorophor BV786; BTLA: Clone JD168-540, Fluorophor PE; all originating from BD Biosciences, San Jose, CA). Cells were pregated for different T-cell populations, and BTLA expression was assessed by fluorescence intensity profiling analyzing mean fluorescence intensities of BTLA staining on the according population. Cytometry analysis was performed using an LSR Fortessa (BD Biosciences, San Jose, CA). Data were then analyzed using the FlowJo 10.6 software (TreeStar, Ashland, OR).

TABLE 1 Patient characteristics

Parameter	Study cohort
Cancer patients	n = 84
Gender [%]:	
Male-female	64.2-35.7
Age (years, median and range)	67.5 (38-87)
BMI (kg/m^2, median and range)	24.1 (15.9-42.3)
Tumor entity (%)	
NSCLC	40.5
Melanoma	13.1
Urogenital tract	13.1
GIT	14.2
Head and neck	10.7
Other malignancies	8.3
Staging (%)	
UICC III	6.2
UICC IV	93.8
ECOG PS (%)	
ECOG 0	7.1
ECOG 1	51.2
ECOG 2	39.3
ECOG 3	2.3
Therapeutic agent (%)	
Nivolumab	59.5
Pembrolizumab	28.5
Nivolumab/Ipilimumab	6.0
Other (avelumab, durvalumab)	6.0
Smoker status (%)	
Never	9.5
Yes, ex	39.2
Yes, present	22.6
Unknown	28.5
Prior therapy (%)	
Yes	71.4
No	28.6
IRAE (%)	
Yes	39.3
No	60.7
IRAE grade III or higher [%]	
Yes	11.9
No	88.1
Healthy controls, n	32

Abbreviations: BMI, body mass index; ECOG PS, Eastern Cooperative Oncology Group's performance status; GIT, gastrointestinal tract, IRAE, immune-related adverse effects; NSCLC, nonsmall cell lung cancer.

2.4 | Statistical analysis

Normal distribution was tested using Shapiro-Wilk test. For the comparison of nonparametric data, Mann-Whitney *U* test and Kruskal-Wallis *H* test were applied. The Spearman correlation coefficient was used for correlation analyses. Box plot graphics demonstrate the median, quartiles and ranges. By plotting the sensitivity against 1 − specificity, receiver operating characteristic (ROC) curves were generated. The influence of a specific parameter on overall survival (OS) was demonstrated using Kaplan-Meier curves. Statistical differences between subgroups were tested applying the log-rank test. To compare differences in sBTLA serum levels between the three longitudinal time points (before ICI treatment, early time point, and late time point), repeated-measures analysis of variance (ANOVA) was applied. The main *F*-test is reported. The Charité cutoff finder, a publicly available software tool, which fits Cox proportional hazard models to the dichotomized survival status (deceased or alive) as well as the survival time (duration between first ICI administration and death/last follow-up) and defines the optimal cutoff as the sBTLA concentration with the most significant split in log-rank test, was used to define optimal cutoff values for the identification of patients with shortened OS.[17] We calculated an individual prognostic cutoff value for each time point of sBTLA measurement (before treatment initialization, at the early/late time point during ICI treatment). To corroborate the prognostic value of variables, univariate and multivariate Cox-regressions were performed. Parameters with a *P* value of <.100 in univariate testing were included in multivariate testing. The hazard ratio (HR) and the 95% confidence interval are displayed. Time-dependent area under the ROC curve (AUC) values were calculated using the "timeROC" package in RStudio (v. 1.2.5033, RStudio Inc., Boston, MA).[18] All statistical analyses were performed using SPSS 23 (SPSS, Chicago, IL).[19] A *P* value of <.05 was considered statistically significant (**P* < .05; ***P* < .01; ****P* < .001).

3 | RESULTS

3.1 | Study population

The study population comprised a total of n = 84 patients with advanced tumor stage (UICC III/IV) treated with ICIs. Table 1 depicts in more detail further cohort characteristics. Patients exhibited a median age of 67.5 years (ranging 38-87 years). Male patients constituted 64.2% of all patients, while females represented 35.8% of the study population. Regarding tumor entity, the most frequent was NSCLC (40.5%), followed by malignant melanoma (13.1%), urogenital cancer (13.1%), gastrointestinal cancers (14.2%), head and neck tumors (10.7%) and others (8.4%), most of which were in a metastasized disease stage UICC IV (93.8%). Concerning toxicity, 39.3% exhibited immune-related adverse effects (IRAE) of any grade, while 60.7% did not.

3.2 | Baseline sBTLA concentrations in cancer patients

Firstly, we compared serum levels of circulating sBTLA in cancer patients and healthy controls in order to get a better perception of the relevance of circulating sBTLA concentrations in patients with advanced cancer stage. We observed significantly elevated levels of sBTLA in serum of cancer patients (median: 215.17 pg/mL) compared to healthy controls (median: 89.70 pg/mL, $P = .027$, Figure 1A). Regarding the discrimination between controls and cancer patients, ROC curve analysis revealed an AUC value of 0.633 for sBTLA (Figure 1B). In addition, in terms of possible confounders, baseline sBTLA levels were comparable between patients with different tumor entities (Supplementary Figure 1A), age group (under or above 67 years old, Supplementary Figure 1B), gender (Supplementary Figure 1C), ECOG performance status (Supplementary Figure 1D), smoker status (Supplementary Figure 1E) and the planned ICI agent (Supplementary Figure 1F). However, we observed significantly higher circulating sBTLA levels in patients with UICC stage IV tumor compared to UICC stage III patients (Supplementary Figure 1G).

3.3 | Baseline sBTLA serum levels do not predict tumor response nor toxicity to ICI therapy

Based on the hypothesis that baseline serum concentrations of sBTLA before therapy with ICIs could help predict if a patient will achieve tumor control and respond to therapy, we compared baseline sBTLA levels between patients who showed a response and those who did not. This evaluation was based on the first-staging CT scan about 3 months after starting the therapy. Despite a trend showing that non-DC patients (n = 45, median sBTLA: 234.61 pg/mL) had higher baseline sBTLA concentrations compared to DC patients (n = 38, median: 164.58 pg/mL, Figure 2A), the difference between both groups was nonsignificant ($P = .157$). This nonsignificant trend toward higher levels of sBTLA in patients categorized as non-DC was confirmed with respect to the individual tumor control at 6 months (DC: median: 164.04 pg/mL, n = 31 vs non-DC: median: 233.40 pg/mL, n = 52, $P = .404$, Figure 2B). Moreover, in order to uncover whether initial sBTLA concentrations can predict if a patient will experience toxicity derived from this treatment, we compared baseline sBTLA levels between patients experiencing IRAE of any degree and patients who did not. In this analysis, there was no significant difference between the two groups (Supplementary Figure 2A). In addition, sBTLA levels were unaltered between patients with grade 3 IRAE or higher and patients with none or less serious IRAE (Supplementary Figure 2B).

3.4 | Baseline sBTLA levels predict OS in patients receiving ICI therapy

Next, we postulated that baseline sBTLA levels might indicate patients' outcome to ICI and compared the OS between different patient groups

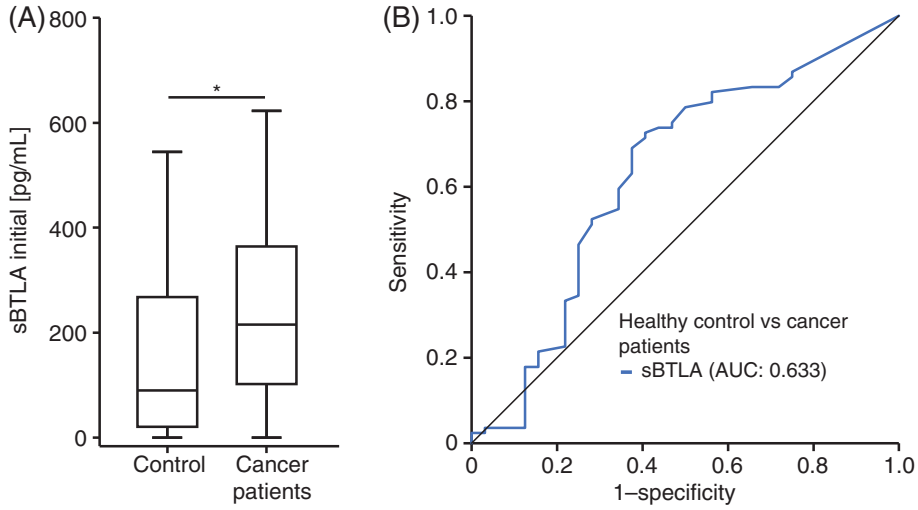

FIGURE 1 sBTLA serum levels are significantly elevated in cancer patients. A, sBTLA serum levels are significantly elevated in cancer patients compared to healthy controls. B, Receiver operating characteristic (ROC) curve analysis reveals an AUC value of 0.633 for sBTLA regarding the discrimination between cancer patients and healthy controls [Color figure can be viewed at wileyonlinelibrary.com]

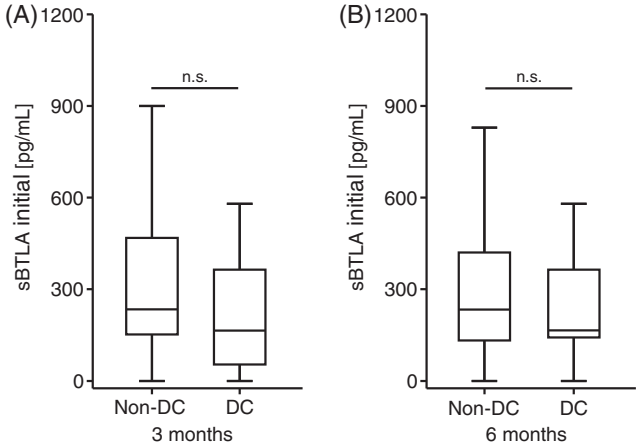

FIGURE 2 Initial sBTLA serum levels do not predict treatment response to ICIs. A, Patients who show disease control (DC) to ICIs at 3 months show a nonsignificant trend ($P = .157$) toward lower baseline sBTLA concentrations compared to non-DC patients. B, Baseline sBTLA levels do not significantly differ between patients with DC at 6 months and non-DC patients

stratified by their baseline sBTLA concentration. At first, we took a look at survival rates at 3 and 6 months and verified that patients who deceased during the first 3 or 6 months after commencing treatment had significantly higher baseline sBTLA concentrations than those who were still alive ($P = .007$ for 3 months, $P = .021$ for 6 months, Figure 3A,B). Next, we applied the median baseline sBTLA concentration (215.17 pg/mL) as a cutoff value and observed that patients with initial sBTLA levels above this value showed a strong trend ($P = .059$) toward an impaired OS compared to patients with lower baseline sBTLA levels (Figure 3C). As the median sBTLA concentration may only act as a suboptimal cutoff value, we next calculated an ideal prognostic cutoff value (see Section 2 for details). This ideal cutoff value (311.64 pg/mL) demonstrated a high prognostic value of baseline sBTLA regarding patients' OS. Patients with an initial sBTLA concentration above this value had a median OS of only 138 days compared to 526 days for patients with initial sBTLA concentrations below the cutoff value (Figure 3D). The sensitivity, specificity, positive predictive value and negative predictive value of the ideal prognostic cutoff value for survival at 3 as well as 6 months were 70.1/73.5%, 37.5/50%, 88.7/67.9% and 33.3/56.7%, respectively. In a further step, we applied univariate and multivariate Cox-regression analyses to identify potential parameters that could also interfere with patients' outcome and therefore affect the prognostic relevance of sBTLA. Baseline sBTLA concentrations above the ideal cutoff value showed a high predictive relevance for OS in univariate analysis (HR: 2.332 [95% CI: 1.377-3.950], $P = .002$, Table 2). In Supplementary Table 1, we depict diverse laboratory markers relevant for this analysis. In the next step. we included parameters with potential prognostic relevance in univariate testing ($P < .100$) into multivariate Cox-regression analysis (Table 2). Here, circulating sBTLA levels above our ideal cutoff value stood out as an independent prognostic parameter for OS (HR: 2.519 [95% CI: 1.457-4.301], $P = .008$, Table 2). Finally, we investigated the prognostic potential of sBTLA in a time-dependent ROC curve analysis as recently described,[18] which revealed that the discriminatory power of sBTLA to distinguish between survivors and nonsurvivors is particularly pronounced during the first 250 days following treatment initialization (Figure 3E).

3.5 | Prognostic relevance of circulating sBTLA at different time points throughout ICI treatment

Since the previous findings demonstrated a clear prognostic value of circulating sBTLA before beginning therapy with ICIs, we next took a closer look at the relevance of longitudinal levels of circulating sBTLA during treatment at an early (n = 70, after one or two cycles) and a late (n = 51, after three, four or five cycles) time point and compared them to baseline values before treatment initialization. The repeated-measures ANOVA analysis including all three time points (before treatment initialization, early time point, late time point) revealed that sBTLA levels did not significantly differ across the three time points (F [2,94] = 0.435, $P = .648$, Figure 4A). Furthermore, we again established ideal prognostic cutoff values for both time points to assess whether circulating sBTLA levels keep their prognostic potential during the course of ICI therapy. Interestingly, for patients with an

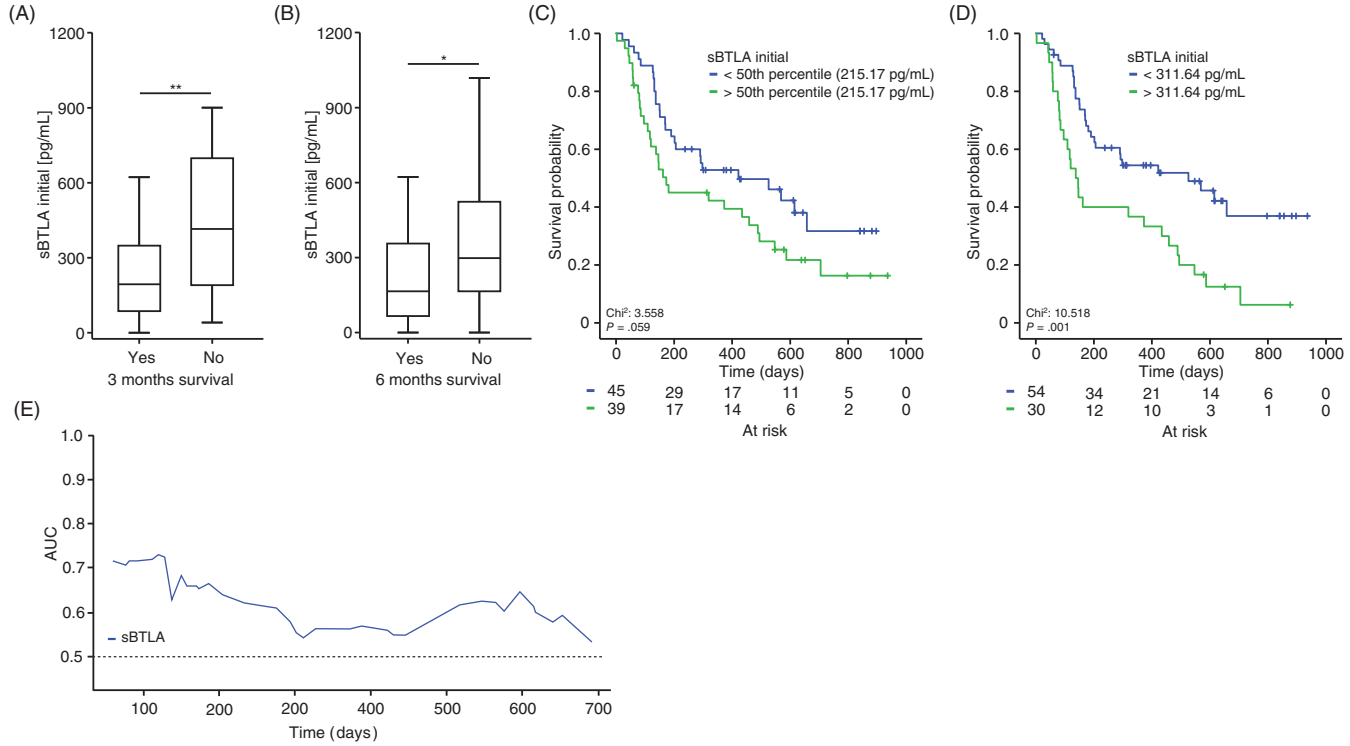

FIGURE 3 Baseline sBTLA levels predict overall survival in patients receiving ICIs. Baseline sBTLA levels are significantly higher in patients who deceased during the first 3 months (A) or 6 months (B) after treatment initialization. C, Using the median sBTLA concentration (215.17 pg/mL) as a cutoff value, patients with high initial sBTLA levels show a strong trend toward a reduced OS compared to patients with low baseline sBTLA levels. D, When applying the optimal cutoff value, patients with a baseline sBTLA concentration above 311.64 pg/mL show a significantly reduced OS with a median OS of just 138 days compared to 526 days for patients with initial sBTLA concentrations below this ideal cutoff. E, Time-dependent ROC curve analysis for the prediction of overall survival [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Univariate and multivariate Cox regression analyses for the prediction of overall survival

	Univariate Cox regression		Multivariate Cox regression	
Parameter	***P* value**	**Hazard ratio (95% CI)**	***P* value**	**Hazard ratio (95% CI)**
BTLA baseline > 311.64 pg/mL	.002	2.332 (1.377–3.950)	.001	2.519 (1.457–4.301)
Age	.679	1.006 (0.979-1.033)		
Sex	.618	0.871 (0.507-1.498)		
UICC tumor stage	.450	1.726 (0.418-7.125)		
ECOG PS	.030	1.613 (1.049-2.482)	.003	2.063 (1.286-3.310)
Leukocyte count	.184	1.038 (0.982-1.097)		
Sodium	.133	0.953 (0.894-1.015)		
Potassium	.837	0.946 (0.558-1.604)		
ALT	.011	1.011 (1.002-1.019)	.036	1.017 (1.001-1.032)
AST	.055	1.010 (1.000-1.021)	.737	0.997 (0.979-1.015)
Bilirubin	.147	1.510 (0.865-2.635)		
Creatinine	.449	0.852 (0.562-1.291)		
LDH	.924	1.000 (0.998-1.002)		

Abbreviations: AST, aspartate transaminase; BMI, body mass index; BTLA, B and T lymphocyte attenuator; ECOG PS, Eastern Cooperative Oncology Group's performance status; LDH, lactate dehydrogenase; UICC, Union for International Cancer Control.

sBTLA concentration above the ideal cutoff values (early time point: 338.74 pg/mL, late time point: 237.39 pg/mL), OS was significantly reduced when compared to patients with lower sBTLA concentrations throughout the course of treatment (Figure 4B,C). The impact of circulating sBTLA concentrations above the ideal cutoff values for both the early and late time points on OS was again corroborated by Cox-

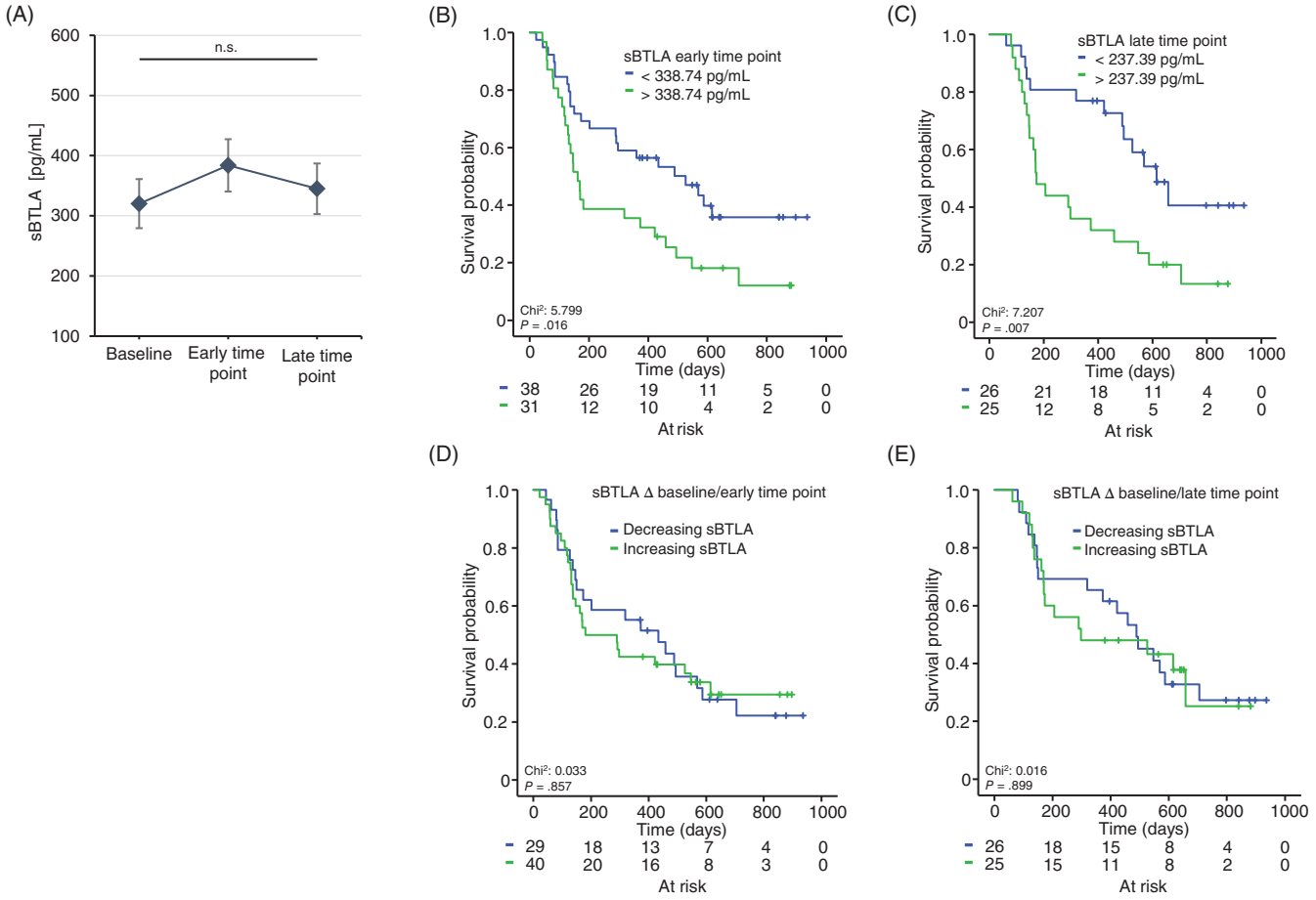

FIGURE 4 Prognostic relevance of circulating sBTLA during ICI treatment. A, Serum sBTLA levels do not significantly change during the course of ICI therapy (error bars indicate SEM. B,C, Patients with sBTLA serum levels above the respective optimal cutoff value (early time point: 338.74 pg/mL, late time point: 237.39 pg/mL) show a significantly impaired OS compared to patients with lower sBTLA levels during the course of ICI treatment. D,E, There is no survival benefit in patients who show increasing or decreasing sBTLA concentrations at the early or late time point compared to baseline levels [Color figure can be viewed at wileyonlinelibrary.com]

regression analyses (HR_{early}: 1.975 [95% CI: 1.123-3.474, $P = .018$; HR_{late}: 2.520 [95% CI: 1.254-5.063, $P = .009$).

In a further step, we assessed the individual dynamic of sBTLA concentrations during the course of ICI therapy regarding the patient's outcome. When comparing the OS of patients showing increasing sBTLA levels between baseline and the early time point to those patients with decreasing sBTLA concentrations, we observed no significant difference of OS (Figure 4D). A similar comparison between the longitudinal dynamic of levels at baseline and the late time point showed no survival benefit for patients with increasing or decreasing sBTLA levels (Figure 4E).

3.6 | Correlation between CD3 + CD8 + BTLA+ peripheral blood mononuclear cells and circulating sBTLA levels

We finally aimed at analyzing a potential correlation between elevated sBTLA levels and high cellular expression levels of BTLA on PBMCs. We used multicolor flow cytometry to evaluate surface expression of BTLA on CTLs, in an exploratory subset of patients (n = 6) before ICI treatment. Flow cytometry data from our analyses, including gating, event count, frequency values for gated CD3 + CD4-CD8 + BTLA+ cell populations and MFI (mean fluorescent intensity), are displayed in Supplementary Table 2. In Supplementary Figures 3A-E, flow histograms depict the BTLA expression within CD3 + CD4- CD8+ cytotoxic T lymphocytes of our six patients. For a better understanding of the gating strategy and the interindividual differences in BTLA expression, we show a comparison between three particular patients, the one with the highest and the lowest BTLA expression in our study population and a patient with an average expression (Supplementary Figure 3E). Interestingly, expression levels of BTLA on the cell surface of CD3 + CD8 + BTLA+ PBMCs significantly correlated with circulating sBTLA concentrations (r: 0.820, $P = .046$, Supplementary Figure 3F).

4 | DISCUSSION

Cancer immunotherapy with ICIs has led to a paradigm shift in the treatment of diverse cancer types, mainly in the advanced stage.[3,4,20] For NSCLC, one of the most frequent cancers in both sexes and the

leading cancer-related death cause,[2] median OS in advanced stages has almost doubled with ICIs compared to platinum-based agents used as a standard of treatment in the past.[4,5] In melanoma patients, a tumor with a low OS when metastatic through use of dacarbazine or temozolomide presently has a 5-year OS of almost 50% when treated with immunotherapy.[3,21] Nonetheless, responses and toxicities still diverge between patients, with some even rather benefiting from conventional chemotherapy.[22] Thus, there is a need for preselection criteria that can accurately predict whether a patient will respond and tolerate this novel therapy. Several studies have explored the immunoregulatory axis of anticancer immunity as means to search for different possible biomarkers to predict response, outcome and toxicity to treatment with these agents, some presenting very interesting biomarker candidates that are being clinically established,[9,23] such as the tumoral PD-L1 expression[24] and the tumor mutational burden.[25] Furthermore, the tumor microenvironment seems to play an important role in modulating the response to ICIs, mainly due to the receptor expression profiles of tumor-infiltrating lymphocytes (TILs).[16,26] Apart from the tumor itself and its immediate milieu, an analysis of peripheral blood, for example, by measuring the neutrophil-to-lymphocyte ratio or ctDNA levels,[27,28] or even an analysis of a stool sample, assessing the individual constitution of the gut microbiome,[29] has demonstrated great potential in helping predict response and outcome of cancer immunotherapy, and may help identify eligible candidates for ICI treatment. Additionally, the surface expression of some immunomodulatory receptors in certain PBMCs, such as elevated expression of T-cell immunoglobulin and mucin domain-containing protein (TIM-3), also seems to play a role in response and outcome prediction.[30] Since many tumors express diverse inhibitory ligands and TILs express multiple coinhibitory receptors, such as BTLA and TIM-3, there are many different pathways that can be targeted as means to enhance antitumor immunity via dual or triple blockade of immune checkpoints with ongoing clinical studies and past trials showing promising results.[31,32] Despite the extensive study of the role of these immune checkpoint molecules, only a reduced number of investigations with limited patient numbers or cancer entities have reported on the relevance of these molecules in their soluble form toward predicting response and outcome to ICI therapy.[14,33] Here, we focused on BTLA (CD272), a coinhibitory immune checkpoint receptor expressed on the surface of T cells, B cells, DCs and myeloid cells. When BTLA binds with its ligand HVEM, it delivers a coinhibitory signal that inhibits T cell response and proliferation.[10] Moreover, dual blockade of BTLA and PD-1 has been shown to enhance immune response against tumors.[31] The soluble form of BTLA (sBTLA) is produced via alternative splicing processes or by cleavage of membrane-bound proteins.[33] In the past, sBTLA has shown a role in the prediction of outcomes in patients experiencing sepsis,[12,34] as well as hepatocellular carcinoma undergoing sorafenib treatment.[35] Therefore, in this study, comprising a series of different tumor entities and checkpoint inhibitors used for therapy, we hypothesized that sBTLA serum levels could serve as a potential biomarker for predicting responses and outcomes in patients receiving ICIs.

To establish a baseline comparison, we verified that circulating values of sBTLA in patients with solid tumors were higher than controls, as shown in the past.[35] Nevertheless, sBTLA levels before initialization of therapy did not show a significant difference between patients responding to therapy and the ones who did not, at both 3 and 6 months after commencing treatment. However, there was a clear trend toward higher sBTLA levels for patients who did not respond to treatment ($P = .117$). Importantly, sBTLA levels, not only prior, but also while on therapy, demonstrated a prognostic relevance in predicting patients' OS. As such, patients with sBTLA serum levels above the optimal cutoff value (baseline: 311.64 pg/mL) had a significantly reduced median OS of only 138 compared to 526 days for patients with a baseline sBTLA concentration above the cutoff. The prognostic relevance of circulating sBTLA was further corroborated by univariate and multivariate Cox-regression analyses, including various clinical and pathophysiological confounders. According to our hypothesis, higher concentrations of circulating sBTLA are the result of cumulative cleavage and alternative splicing processes derived from a higher expression of BTLA in the surface of T cells and hence possibly in TILs. To corroborate our assumption that sBTLA levels are associated with BTLA expression, we analyzed surface expression of BTLA on peripheral T cells in a small subset of six patients. In this case and despite the low number of samples, we found a significant correlation between levels of circulating sBTLA and levels of CD3 + CD8 + BTLA+ T cells in peripheral blood, further sustaining our postulate (see Supplementary Figure 3).

Considering the known coinhibitory immunomodulatory effects of BTLA in TILs,[16,32] higher expression of this molecule on the surface of T cells ultimately leads to a higher degree of T-cell inhibition and anergy and the immune system's incapability of fighting the tumor, resulting subsequently in an impaired survival. Ultimately, these observations verify the thesis that BTLA is yet another potential target in the immense landscape of future checkpoint inhibitor targets, enhancing antitumor immunity when blocked.[36] Studies exploring the rationale for the potential clinical use of BTLA in combination or by itself in cancer immunotherapy have shown promising results,[37,38] so that in April 2019 the first monoclonal antibody against BTLA was approved by the FDA for drug trial.[39] Regarding a direct inhibition of the soluble form of this molecule, sBTLA, this is certainly a possibility worth exploring; however, further studies characterizing its precise role in anticancer immune response are warranted. On the one hand, sBTLA could, likewise a comparable coinhibitory soluble immune checkpoint molecule sPD-1, competitively bind its direct ligand in antigen presenting cell (APCs; for sPD-1 being PDL-1, for BTLA HVEM), enhancing antitumor responses. However, on the other hand, it could also mimic the effects of sCTLA-4, which has been shown to have strong inhibitory properties and its inhibition could lead to increased antitumor immunity.[32]

It is difficult to assume that in the future a patient will be elected for ICI therapy based only on a low pretherapeutic serum concentration of sBTLA. Nevertheless, the detection of this molecule may play a part in a more extensive peripheral immunome screening, eventually involving more soluble forms of other checkpoint inhibitors, be it of

the coinhibitory variant, such as the T-cell immunoreceptor with Ig and ITIM domains (TIGIT) or the lymphocyte activation gene 3 (LAG-3), or of the costimulatory variant, such as CD27 and OX40.[40] These could represent feasible, cost-effective and noninvasive biomarkers to help electing eligible candidates to ICI therapy.

Our study does have some limitations. First, our cohort includes a very heterogeneous population comprising several tumor entities and different immune checkpoint therapy agents. The single-center design allows a solid comparability of the heterogeneous study population with respect to specific demographic characteristics and different laboratorial parameter values, but does clearly warrant a confirmatory multicenter approach. Furthermore, the basket design demonstrated, in contrast to many biomarker studies including patients from a specific tumor entity being treated with a specific checkpoint inhibitor, that sBTLA has the potential to serve as a therapy outcome prediction tool for ICI therapy, regardless of the tumor and the planned checkpoint inhibitor agent (see Supplementary Figure 1). Nevertheless, we believe that it is important to point out that, given the heterogeneity of our patient collection and that different tumor entities react differently to ICI therapy, we cannot sufficiently answer the question in which tumor entity the prognostic relevance of sBTLA is the highest. Simultaneously, it is important to acknowledge that we did not include alternative treatment approaches such as chemotherapy or radiotherapy, but only investigated patients undergoing ICI treatment. Therefore, we cannot postulate that a tumor patient with an initial (or even longitudinal) sBTLA serum level above our ideal prognostic cutoff value might have had a similar or even better outcome in case of a different treatment. Additionally, it is noteworthy that the correlation analysis between BTLA expression of PBMCs and circulating levels of sBTLA is based on a very small cohort of patients ($n = 6$), and despite its encouraging results, it warrants further confirmation in larger patient collectives, eventually even including BTLA expression in TILs of tumor samples.

In conclusion, although confirmatory multicenter trials, ideally including different treatment modalities and other soluble forms of immune checkpoints, are warranted to gain full insight into the role of sBTLA as a novel biomarker before and during ICI therapy, our study provides evidence that elevated levels of sBTLA are associated with an impaired OS of patients undergoing ICI therapy, which might help to select the most adequate candidates for this cancer immunotherapy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

The study protocol was approved by the ethics committee of the University Hospital RWTH Aachen, Germany (EK 206/09) and conducted in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from the patients.

DATA AVAILABILITY STATEMENT

Data will be made available, upon reasonable request, by the corresponding author.

ORCID

Joao Gorgulho https://orcid.org/0000-0003-1986-8208
Sven H. Loosen https://orcid.org/0000-0003-3447-1161

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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