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ORIGINAL ARTICLE

Baseline extracellular vesicle TGF-β is a predictive biomarker for response to immune checkpoint inhibitors and survival in non–small cell lung cancer

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Abstract

Background: Immune-checkpoint inhibitors (ICIs) are an effective therapeutic strategy, improving the survival of patients with lung cancer compared with conventional treatments. However, novel predictive biomarkers are needed to stratify which patients derive clinical benefit because the currently used and highly heterogenic histological PD-L1 has shown low accuracy. Liquid biopsy is the analysis of biomarkers in body fluids and represents a minimally invasive tool that can be used to monitor tumor evolution and treatment effects, potentially reducing biases associated with tumor heterogeneity associated with tissue biopsies. In this context, cytokines, such as transforming growth factor-β (TGF-β), can be found free in circulation in the blood and packaged into extracellular vesicles (EVs), which have a specific delivery tropism and can affect in tumor/immune system interaction. TGF-β is an immunosuppressive cytokine that plays a crucial role in tumor immune escape, treatment resistance, and metastasis. Thus, we aimed to evaluate the predictive value of circulating and EV TGF-β in patients with non–small-cell lung cancer receiving ICIs.

Methods: Plasma samples were collected in 33 patients with advanced non–small-cell lung cancer before and during treatment with ICIs. EV were isolated from plasma by serial ultracentrifugation methods and circulating and EV TGF-β expression levels were evaluated by enzyme-linked immunosorbent assay.

Results: Baseline high expression of TGF-β in EVs was associated with nonresponse to ICIs as well as shorter progression-free survival and overall survival, outperforming circulating TGF-β levels and tissue PD-L1 as a predictive biomarker.

Conclusion: If validated, EV TGF-β could be used to improve patient stratification, increasing the effectiveness of treatment with ICIs and potentially informing combinatory treatments with TGF-β blockade.

Plain language summary

- Treatment with immune-checkpoint inhibitors (ICIs) has improved the survival of some patients with lung cancer. However, the majority of patients do not benefit from this treatment, making it essential to develop more reliable biomarkers to identify patients most likely to benefit.
- In this pilot study, the expression of transforming growth factor-β (TGF-β) in blood circulation and in extracellular vesicles was analyzed.
- The levels of extracellular vesicle TGF-β before treatment were able to determine which patients would benefit from treatment with ICIs and have a longer survival with higher accuracy than circulating TGF-β and tissue PD-L1, which is the currently used biomarker in clinical practice.

KEYWORDS
biomarker, extracellular vesicles, immunotherapy, non-small cell lung cancer, TGF-β

BACKGROUND

Immune checkpoint inhibitors have improved the outcomes of patients with non–small-cell lung cancer (NSCLC), becoming the standard of care in multiple lines of therapy.[1] However, only a subset of these patients experiences durable clinical benefit, underscoring the need to develop more reliable predictive biomarkers for patient stratification. To date, tissue PD-L1 is the most commonly used biomarker, but it is associated with high intra- and intertumoral variability. Thus, there is significant room for improvement in predictive accuracy for this drug class.[2,3]

When searching for an optimal biomarker, it is crucial to understand the high intratumoral and intrapatient heterogeneity observed in lung carcinomas.[4] As a key example, expression of PD-L1 or other molecules may vary according to the biopsied area of the tumor, the primary or metastatic lesion, as well as the time of tumor collection. Because immune-checkpoint inhibitors (ICIs) may be administered after several lines of therapy, the levels of the specific biomarker may have been altered by treatment, degree of inflammation, or epigenetic and other changes in the tumor microenvironment that evolve over time.[5] The limitations of PD-L1 tissue expression as a biomarker highlight the importance of using minimally invasive and repeatable biomarkers, such as liquid biopsy biomarkers in blood, to monitor changes in tumor evolution, potentially even in the tumor microenvironment in real time.[6]

In this context, changes in cytokine levels have been described to regulate the tumor microenvironment and to be associated with response to ICIs in some pan-cancer studies.[7,8] In particular, transforming growth factor-β (TGF-β) is an extensively studied cytokine in cancer because it has been found to be overexpressed in tumors and the tumor microenvironment in comparison to nontransformed tissues.[9] TGF-β plays a significant role in the tumor progression and metastasis because it contributes to the activation of the epithelial-to-mesenchymal transition, tumor growth, invasion, extracellular matrix remodeling, angiogenesis, and immunosuppression.[10,11] Moreover, TGF-β is highly implicated in immune modulation and tumor immune escape by exerting direct and indirect immunosuppressive activities. For example, cancer-associated fibroblasts secrete TGF-β, which promotes tumor progression by inducing the differentiation of naïve CD4+ T cells into regulatory T cells and T-helper type 17 cells.[12,13] Myeloid-derived suppressor cells can also release this cytokine-suppressing T and natural killer cell activation. Thus, the TGF-β level is a promising biomarker candidate for monitoring responses to ICIs.

In addition to being released into circulation, cytokines can be selectively incorporated into extracellular vesicles (EVs) in response

to specific stimuli,[14] thereby being protected from degradation during circulation, and being released to specific cells in a process regulated by EV tropism.[15,16] Interestingly, a recent study demonstrated that TGF-β in the tumor microenvironment orchestrated PD-L1 enrichment into tumor-derived EVs and promoted CD8+ T-cell dysfunction.[17] This suggests a shared role between these two molecules in modulating the immune response. In addition, EVs play a role in the immune response and can contain both tumor antigens and immunosuppressive mediators.[9]

To date, several pan-cancer studies have evaluated the impact of cytokines, including TGF-β, on the response to immunotherapy and patient survival.[18] However, most of these studies evaluated histological samples and no study has evaluated the role of circulating and EV TGF-β in response to immunotherapy in patients with NSCLC. Moreover, there is compelling evidence that the antitumor immune response is a complex process regulated by the interaction between the tumor, the immune system, and multiple host factors in which both cytokines and EVs play a critical role. Indeed, most cells present in the tumor microenvironment can be influenced by TGF-β levels but may develop responses involving heterogeneous pathways. Thus, to fully understand the role of TGF-β and fill this gap in knowledge, we undertook a pilot study to evaluate both circulating and EV forms present in the plasma, released by cancerous and noncancerous cells, that may better represent the complex interplay that takes place in the tumor microenvironment.[18]

MATERIALS AND METHODS

Patients and samples

A retrospective cohort of patients with advanced/metastatic NSCLC treated with pembrolizumab/nivolumab at the Azienda Ospedaliera Universitaria Policlinico "G. Martino," Messina, Italy, were included in this study (Figure 1). Institutional review board approval was received and written informed consent was signed by every patient. Further information from these patients regarding follow-up and selection criteria can be found in our previous study.[19] Tissue PD-L1 tumor proportion score (TPS) was measured by standard immunohistochemistry from the primary lung tumor or metastasis. Durable treatment response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1[20] at 21 ± 3 weeks during treatment.

Blood samples and cytokine characterization

Blood samples were collected before treatment (T1) and at week 9 ± 1 during treatment (T2). Plasma was obtained after centrifugation at 2000*g* for 15 min and immediately frozen at −80°C until further processing. Plasma aliquots were thawed and centrifuged at 3000*g* for 20 min at 4°C. Supernatants were collected and 100 μl were used for

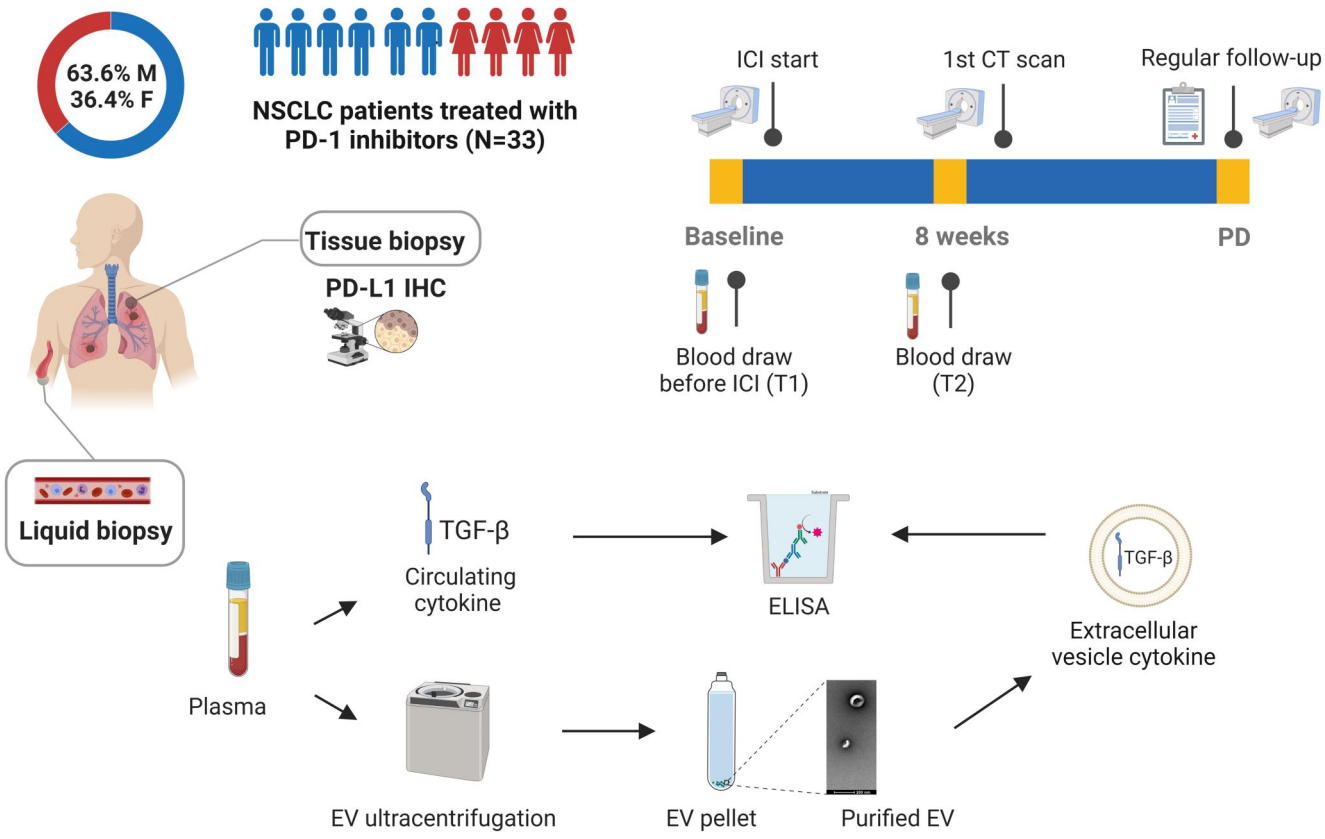


FIGURE 1 Study design and cytokine analysis. [Created with Biorender.com]

the analysis of circulating TGF-β, whereas the remaining volume was used for EV isolation as previously reported in our first analysis of these patients.[19] EVs were lysed with 1X RIPA lysis buffer (Cell Signaling) and sonicated for 2 min before EV-encapsulated cytokine analysis. Circulating and EV TGF-β-1 levels were measured in plasma and lysed EVs with the Milliplex Multiplex Assay (Millipore Sigma) following manufacturer's instructions in a Luminex MagPix using Luminex's exponent software. The full characterization of EV size, concentration, and protein markers from these samples was performed following the standardized protocol from our group[19] according to the latest recommendations of the International Society of Extracellular Vesicles.[21]

TGF-β-1 spiking controls

One potential concern of this study was the purity and origin of the TGF-β-1 found in EV from plasma because centrifugation protocols could theoretically inadvertently incorporate the coprecipitation of protein complex and aggregates of circulating cytokines. For this reason, we performed spike controls with TGF-β-1. Plasma samples from three patients were divided into four aliquots each, two parts for circulating TGF-β-1 and two for EV TGF-β-1 analyses. Then, 40,000 pg/ml of human TGF-β-1 recombinant protein (Peprotech) were added to one of the circulating and one of the EV aliquots and compared with the respective negative control from the same patient. Circulating cytokines and EV-contained cytokines were processed as previously described.

Statistical methods

Graphs and statistical analysis were performed using SPSS v.22.0 (IBM Corp.) and GraphPad Prism Version 8.4 (GraphPad Software Inc.). Nonparametric tests were used to compare variables (Mann-Whitney *U*, Kruskal–Wallis, Wilcoxon matched-pair signed rank, Spearman rank correlation, and Fisher exact tests). Logistic binary regression was used to create receiver-operating characteristic curves and the area under the curve (AUC) was calculated to evaluate the sensitivity and specificity of each model. Circulating and EV TGF-β cutoffs were selected to maximize progression-free survival (PFS) and overall survival (OS) predictive value. This resulted in a cutoff of 28,820 pg/ml for circulating TGF-β and 667.5 pg/ml for EV levels. Survival was analyzed by Kaplan–Meier (log-rank test) and Cox proportional-hazards regression. Two-tailed $p < .05$ was considered statistically significant.

RESULTS

Thirty-three patients diagnosed with advanced/metastatic NSCLC undergoing treatment with ICIs were enrolled in this study, with a median follow-up of 12.4 months (range, 2.5–33.1). Clinical characteristics of these patients can be found in Table 1.

Circulating and EV TGF-β characterization

The results of the spiking controls showed that the addition of TGF-β to the plasma highly increased the circulating levels ($p = .017$) but not those in EVs ($p = .269$), dismissing potential concerns for co-isolation contamination (Figure S1). Both forms of this cytokine were analyzed in paired plasma samples from the 33 patients. Circulating and EV TGF-β levels were positively correlated before treatment (T1) ($p = .002$) but not during treatment (T2) ($p = .296$) (Figure 2A). Moreover, no significant net changes in the levels of circulating or EV TGF-β were observed during the treatment in the population ($p = .688$ and $p = .437$, respectively) (Figure 2B).

Circulating and EV TGF-β and clinical characteristics

Analysis of TGF-β levels and their correlation with the clinical characteristics are found in Table S1. Significantly, higher EV TGF-β (T2) levels were found in patients with high tissue PD-L1 expression ($p = .027$) and those receiving pembrolizumab ($p = .036$) and first-line ICIs ($p = .015$). However, no significant changes in circulating or EV TGF-β during treatment were found in these groups ($p > .05$). In addition, no specific associations were found between EV TGF-β (T1), circulating TGF-β, and any clinical characteristics.

High EV TGF-β is associated with nonresponders to ICIs

From the 33 patients with NSCLC who received ICI treatment, 15 (45.5%) were classified as durable responders and the other 18 (54.5%) as nonresponders. Nonresponders showed higher levels of EV TGF-β (T1) than responders ($p = .047$), whereas nonsignificant differences were observed regarding circulating TGF-β (T1) ($p = .563$) (Figure 3A). No relation was found between the treatment response to ICIs and cytokine levels during treatment (T2) or with the dynamics or trajectory of these cytokines over the course of treatment (T1-T2) ($p > .05$).

Moreover, the predictive value of the current standard-of-care tissue PD-L1 TPS was analyzed and demonstrated no association with treatment response ($p = .448$). As observed in the receiver-operating characteristic curve, the EV TGF-β (T1) was the best predictor for durable response with an AUC = 70.4%, whereas the circulating version and the tissue PD-L1 only showed 55.9% and 62.6%, respectively (Figure 3B,C). When maximizing high specificity of 86.7%, EV TGF-β (T1) identified nonresponders with a higher sensitivity (44.4%) and accuracy (63.6%) than the circulating TGF-β or tissue PD-L1 TPS (Figure 3B,C). Subgrouped analysis of the predictive performance of EV TGF-β (T1) across different types and lines of therapy is shown in Figure S2. On the other hand, when EV TGF-β (T1) expression levels and the standard-of-care tissue PD-L1 were combined, only a slight increase in accuracy (66.7%) was observed (Figure S3).

TABLE 1 Characteristics of the NSCLC populations

Characteristics	ICIs (33) Patients, No. (%)
Sex	
Men	21 (63.6)
Women	12 (36.4)
Age (years)	
Mean ± standard deviation	68.1 ± 11.3
Smoking habits	
Never smoker	5 (15.2)
Former smoker	18 (54.5)
Current smoker	10 (30.3)
Histological subtype	
Non-SCC	25 (75.8)
SCC	8 (24.2)
Stage	
IV	32 (97.0)
IIIB	1 (3.0)
Immunotherapy treatment	
Pembrolizumab	20 (60.6)
Nivolumab	13 (39.4)
Line	
First	16 (48.5)
Second	15 (45.5)
Third	2 (6.1)
Tissue PD-L1 (TPS)	
Negative (<1%)	5 (15.2)
Low expression (1%–49%)	6 (18.2)
High expression (>50%)	17 (51.5)
Unknown	5 (15.2)
Durable response	
Partial response	4 (12.1)
Stable disease	11 (33.3)
Progressive disease	18 (54.6)
Progression	
Yes	28 (84.8)
No	5 (15.2)
PFS (months)	
Median (range)	5.3 (1.7–27.7)
Death	
Yes	19 (57.6)
No	14 (42.4)
OS (months)	
Median (range)	12.4 (2.5–33.1)

Abbreviations: ICI, immune-checkpoint inhibitor; NSCLC, non–small-cell lung cancer; OS, overall survival; PFS, progression-free survival; SCC, squamous cell carcinoma; TPS, tumor proportion score.

High baseline EV TGF-β is associated with shorter PFS and OS

Twenty-eight (84.8%) patients progressed and 19 (57.6%) died during the follow-up period of this study. Patients with high EV TGF-β (T1) experienced both shorter PFS (hazard ratio [HR], 0.23; $p = .037$) and OS (HR, 0.19; $p = .020$), whereas patients with high levels of circulating TGF-β (T1) only experienced shorter OS (HR, 0.15; $p = .011$) but not PFS (HR, 0.53; $p = .465$) (Figure 4). Similarly, the Cox regression analysis showed that patients with low EV TGF-β (T1) experienced longer PFS (HR, 0.45; $p = .046$) and OS (HR, 0.35; $p = .026$). Low circulating TGF-β (T1) (HR, 0.30; $p = .016$) was also associated with better OS (Table S2).

DISCUSSION

Tumor inflammation can result in the release of tumor-promoting signals that cause the recruitment of specific subtypes of immune cells in the tumor. Thus, cells such as neutrophils, CD4+ regulatory T cells, tumor-associated macrophages, or myeloid-derived suppressor cells have been described as important promoters of a protumoral microenvironment. These immune cells, along with the tumor cells, can secrete cytokines such as TGF-β that activate protumorigenic inflammatory pathways in the tumor microenvironment.[13]

To date, several multicancer studies have analyzed the role of TGF-β and other cytokines as biomarkers for response to immunotherapy and survival. However, most of these studies have focused on histological levels of these cytokines.[18] On the other hand, fewer studies have evaluated EV cytokine expression[15,22–24] and, to the best of our knowledge, none has evaluated the predictive value of circulating and EV cytokines in the response to immunotherapy in patients with NSCLC.

In our study, we first demonstrated that our selected methodology was appropriate to evaluate EV and circulating plasma levels of TGF-β because spiking control experiments showed no significant cross-contamination from cell-free circulating TGF-β during EV ultracentrifugation. This finding concur with previous reports that showed that ultracentrifugation methods yielded purer EVs with more appropriate sizes and lower protein contamination in comparison to precipitation kits and column-affinity methods.[15,25] In

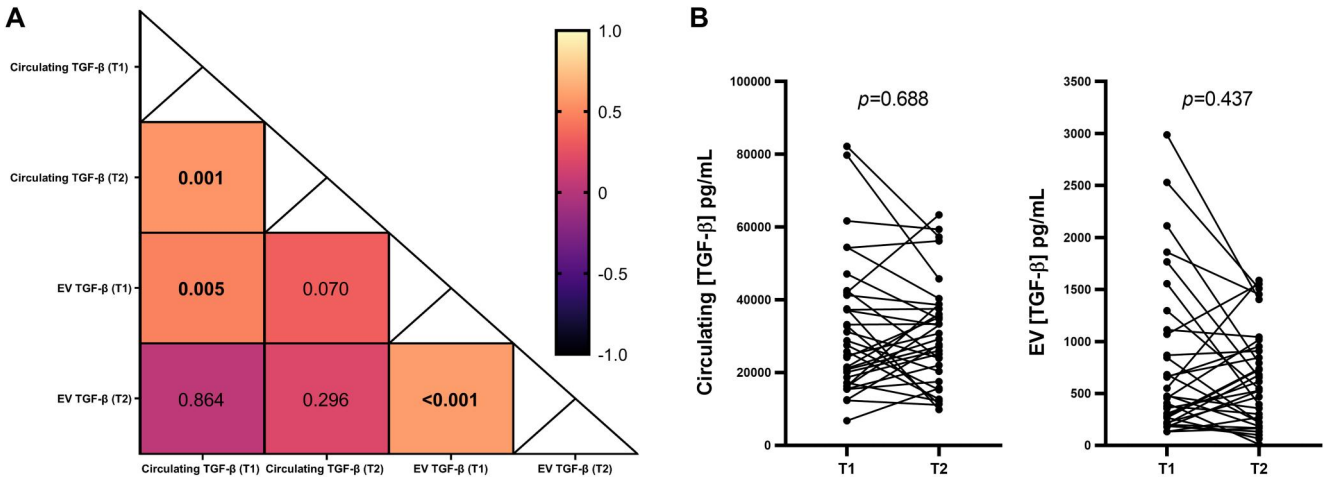
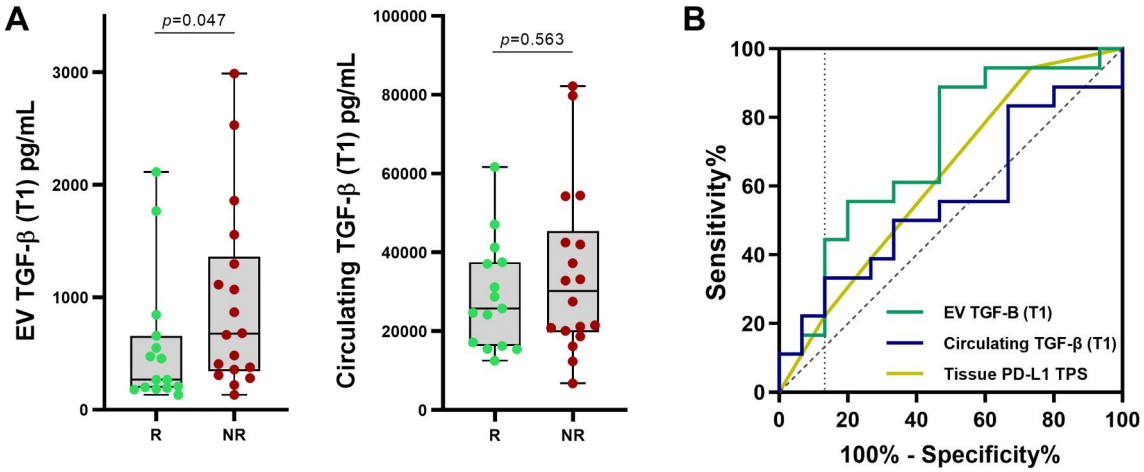


FIGURE 2 Circulating and EV TGF-β expression. (A) Circulating and EV levels of TGF-β were positively correlated at baseline (T1) ($p = .005$) but not during treatment (T2) ($p = .296$). Moreover, EV TGF-β levels at T1 were associated with those at T2 ($p < .001$) and circulating levels at T1 were statistically associated with T2 ($p = .001$) (colors express the grade of correlation, whereas numbers represent p values). (B) No statistical changes were observed in the dynamics of TGF-β in paired samples during ICI treatment. EV indicates extracellular vesicle; ICI, immune-checkpoint inhibitor; TGF-β, transforming growth factor β



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Biomarker	AUC	Sensitivity	Specificity	Accuracy
EV TGF-B (T1)	70.4%	44.4%	86.7%	63.6%
Circulating TGF-β (T1)	55.9%	33.3%	86.7%	57.6%
Tissue PD-L1 TPS	62.6%	22.2%	86.7%	51.5%

FIGURE 3 EV TGF-β outperformed circulating TGF-β and tissue PD-L1 as a predictor of ICIs durable response: (A) Nonresponders (NR) showed higher pretreatment (T1) levels of EV TGF-β than responders (R) ($p = .047$) but not of circulating TGF-β ($p = .563$). (B, C) EV TGF-β (T1) was a better predictor for response than circulating TGF-β and tissue PD-L1 TPS with an AUC = 70.4% vs. 55.9% and 62.6%, respectively. When maximizing specificity at 86.7%, the EV TGF-β showed higher sensitivity and accuracy at predicting nonresponses. AUC indicates area under the curve; EV, extracellular vesicle; ICI, immune-checkpoint inhibitor; TGF-β, transforming growth factor β; TPS, tumor proportion score

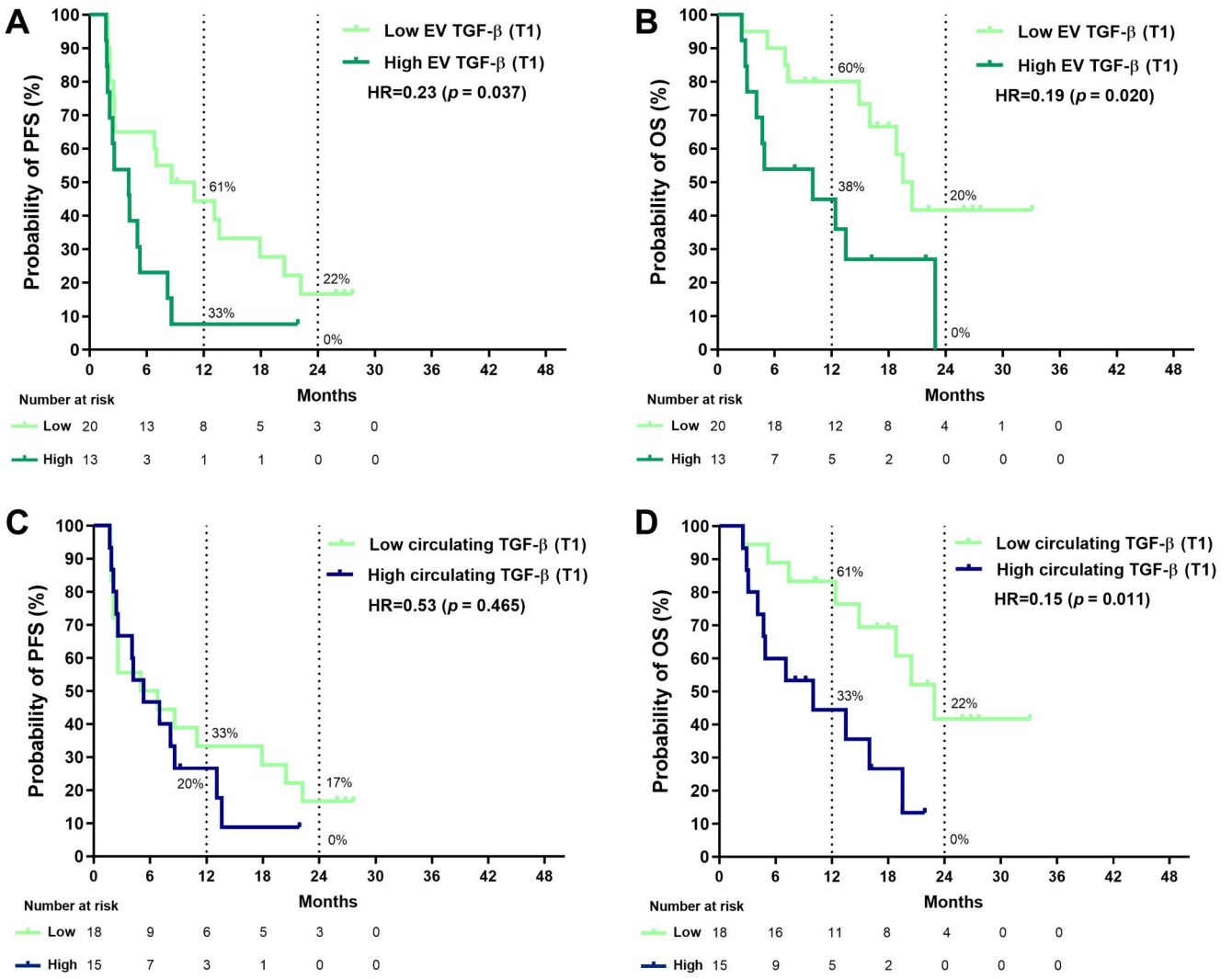

FIGURE 4 EV and circulating TGF-β as a predictive biomarker for PFS and OS. Patients with high EV TGF-β (T1) (dark green) showed shorter PFS ($p = .037$) (A) and OS ($p = .020$) (B). High levels of circulating TGF-β (dark blue) were not associated with PFS ($p = .465$) (C) but they were with shorter OS ($p = .011$) (D). Number of patients at risk every 6 months and the percentage patients free of progression or death at 12 and 24 months have been represented underneath the *x*-axis. EV indicates extracellular vesicle; OS, overall survival; PFS, progression-free survival; TGF-β, transforming growth factor β

addition, whereas other cytokines may be preferentially enriched in the EV surface, TGF-β is found encapsulated within EVs, supporting the need to lyse EVs to effectively analyze the levels of this cytokine.[15]

After validating our methodology, we analyzed the levels of EV and circulating TGF-β before treatment with ICIs and at 8 weeks during treatment in our cohort of 33 patients with NSCLC. We observed that whereas nonresponders presented higher pretreatment levels of EV TGF-β than responders, circulating levels of TGF-β were not associated with treatment response. To evaluate the potential clinical application of TGF-β as a biomarker, we compared it with the current standard-of-care tissue PD-L1 scoring system. Baseline EV TGF-β, with an AUC of 70.4%, showed higher predictive accuracy than either the circulating TGF-β form or the standard tissue PD-L1 diagnostics. Interestingly, when the specificity was maximized to reduce false positives, EV TGF-β presented twice the sensitivity than the tissue PD-L1 (44.4% vs 22.2%, respectively). Similar results were observed when evaluating the predictive role of these biomarkers in foreseeing survival. Low levels of EV TGF-β (T1) were present in patients with longer PFS (HR, 0.45; $p = .046$) and OS (HR, 0.35; $p = .026$). Moreover, low circulating TGF-β (T1) was found in patients with longer OS (HR, 0.30; $p = .016$), but not PFS, and tissue PD-L1 did not show any correlation with either of these outcomes. Additionally, the addition of tissue PD-L1 data to the EV TGF-β (T1) model only slightly increased the accuracy for predicting treatment response.

These observations align with the described immunosuppressive role of this cytokine as TGF-β signaling has been associated with inhibition of tumor infiltrating T cells, lack of response to ICIs, and worse prognosis in NSCLC and other cancers.[5,26–28] Our observations are also supported by the reported higher expression of EV TGF-β in patients with lymph node metastasis and its positive effect

on the induction of regulatory T-cell differentiation, cancer-associated fibroblast activation, and the promotion of an immunosuppressive microenvironment.[23,29,30]

The higher predictive value for treatment response and PFS of EV TGF-β compared with circulating TGF-β could be related to different cellular mechanisms of release and intake. First, in the cell, cytokines may be subjected to a specific mechanism of encapsulation into EVs and subsequent release.[14–16] In addition, EV TGF-β can be retained in the endosomal compartments during cellular uptake, delaying lysosomal degradation and prolonging cellular signaling.[31] Moreover, EV and circulating TGF-β may have different effects on target cells. For example, the EV version has been reported to induce myofibroblast differentiation into proangiogenic and tumor-promoting myofibroblast, whereas the circulating version has not, suggesting that EV TGF-β may be necessary for the formation of the tumor-promoting stroma.[32] Thus, the EV TGF-β may better reflect the status of the tumor microenvironment or have a more important role in the development of treatment resistance than the circulating free form, which may predominantly be produced during cellular apoptosis.[33]

Moreover, pretreatment levels of EV TGF-β presented superior predictive value than tissue PD-L1. This is not surprising because of several limitations associated with this biomarker, including spatial and temporal heterogeneity or threshold and assay variability that have caused this biomarker to be predictive in only 28.9% of pan-cancer cases.[34] This observation highlights the importance of the implementation of noninvasive and repetitive biomarkers such as EV TGF-β characterization that could be used to aid in patient stratification and ultimately improving survival rates.

A correlation was observed between higher levels of EV TGF-β during treatment and higher tissue PD-L1 expression, which was also related to the type and line of treatment received. This could be related to the reported activation of the epithelial-to-mesenchymal transition by high levels of tissue PD-L1 that could, in turn, lead to higher levels of TGF-β.[35] Nonetheless, no significant increases in TGF-β were found regarding tissue PD-L1 expression or type of treatment.

Over the years, several preclinical and clinical studies have evaluated the efficacy of TGF-β blockade in monotherapy or in combination with other treatments.[9] In particular, TGF-β inhibition has been shown to potentiate the effect of ICIs in unresponsive tumors[36] through a variety of potential mechanisms, including inhibiting epithelial-mesenchymal transition, enhancing the activity of natural killer and cytotoxic T cells, reducing the suppressive activity of T-regulatory cells, and increasing the antitumor activity of other agents.[37]

Furthermore, in early clinical trials, TGF-β pathway antagonists such as galunisertib or vactosertib have shown promising results as therapeutic agents considering their safety profile, but further clinical trials are needed.[38] Similarly, the use of Bintrafusp alfa, a PD-L1 and TGF-β dual inhibitor, provided encouraging results in patients with advanced NSCLC after platinum treatment.[39] However, it has shown contradictory results in still-ongoing trials when compared with pembrolizumab administered in the first line.[40] Because of the lack of predictive biomarkers for these novel therapies, the use of EV TGF-β could be key to patient stratification, maximizing the effectiveness of TGF-β inhibitors as single-agents or in combination with ICIs, and accelerating the clinical application of these promising treatments. Limitations of this study include its preliminary nature with small sample size and heterogeneity of treatments, requiring validation in larger cohorts. We showed the subgrouped analysis of the performance of EV TGF-β as a predictor of durable response in different lines and treatments; however, no conclusions can be derived from them because low number of patients were included in each group. These intriguing data are suggested as hypothesis generating as opposed to hypothesis confirming. Furthermore, the relative prognostic effect of elevated TGF-β cannot be strictly isolated in this data set.

CONCLUSION

Despite the preliminary nature of this work including a small cohort of patients, we showed, for the first time, evidence that suggests EV TGF-β expression is a better predictor for treatment response and survival than circulating TGF-β and tissue PD-L1 in patients with NSCLC who are receiving ICIs. These results lay the foundation for the inclusion of EV TGF-β as a potential predictive biomarker in immunotherapy studies including dual PD-(L)1 and TGF-β inhibition.

AUTHOR CONTRIBUTIONS

Diego de Miguel-Perez: Formal analysis and methodology, EV isolation and characterization, cytokine analysis, statistical analysis and visualization, investigation, writing – original draft. **Alessandro Russo:** Data curation and patient recruitment, statistical analysis and visualization, investigation, and writing – original draft. **Muthukumar Gunasekaran:** Formal analysis and methodology, EV isolation and characterization, and investigation. **Francesco Buemi:** data curation and patient recruitment. **Lisa Hester:** Cytokine analysis. **Xiaoxuan Fan:** Cytokine analysis. **Brandon A. Carter-Cooper:** Formal analysis and methodology, EV isolation and characterization. **Rena G. Lapidus:** Formal analysis and methodology and EV isolation and characterization. **Ariel Peleg:** Investigation. **Marisol Arroyo-Hernández:** Investigation. **Andres F. Cardona:** Investigation. **Aung Naing:** Investigation. **Fred R. Hirsch:** Investigation. **Philip C. Mack:** Investigation. **Sunjay Kaushal:** Investigation. **Maria Jose Serrano:** Investigation. **Vincenzo Adamo:** Data curation and patient recruitment. **Christian Rolfo:** Conceptualization and writing – original draft. All authors reviewed, read, and approved the final version of the manuscript.

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CONFLICT OF INTEREST

Alessandro Russo reports advisory board role/consultancy for AstraZeneca, Novartis, and MSD outside the submitted work. Andres F. Cardona discloses financial research support from Merck Sharp & Dohme, Boehringer Ingelheim, Roche, Bristol-Myers Squibb, Foundation Medicine, Roche Diagnostics, Thermo Fisher, Broad Institute, BioNTech, Amgen, Flatiron Health, Teva Pharma, Rochem Biocare, Bayer, INQBox and The Foundation for Clinical and Applied Cancer Research – FICMAC; advisor role to EISAI, Merck Serono, Jannsen Pharmaceutical, Merck Sharp & Dohme, Boehringer Ingelheim, Roche, Bristol-Myers Squibb, Pfizer, Novartis, Celldex Therapeutics, Foundation Medicine, Eli Lilly, Guardant Health, Illumina, and Foundation for Clinical and Applied Cancer Research – FICMAC. Aung Naing discloses research funding from NCI, EMD Serono, MedImmune, Healios Onc. Nutrition, Atterocor/Millendo, Amplimmune, ARMO BioSciences, Karyopharm Therapeutics, Incyte, Novartis, Regeneron, Merck, Bristol-Myers Squibb, Pfizer, CytomX Therapeutics, Neon Therapeutics, Calithera Biosciences, TopAlliance Biosciences, Eli Lilly, Kymab, PsiOxus, Arcus Biosciences, NeoImmuneTech, ImmuneOncia, Surface Oncology, Monopteros Therapeutics, BioNTech SE, Seven & Eight Biopharma, and SOTIO Biotech AG; advisory board activity for CytomX Therapeutics, Novartis, Genome & Company, OncoSec KEYNOTE-695, Kymab, STCube Pharmaceuticals, and Deka Biosciences; he also reports an advisory board role for Takeda, CSL, Behring, Horizon, and Pharming; travel and accommodation expense from ARMO BioSciences: Spouse and research funding from Immune Deficiency Foundation, Jeffery Modell Foundation and chao physician-scientist, and Baxalta. Fred R. Hirsch reports advisory boards consultancy for Bristol-Myers Squibb, AstraZeneca/Daiichi, Sanofi/Regeneron, Novartis, Amgen, OncoCyte, Genentech, and Nectin Therapeutics. Christian Rolfo is a speaker for Merck Sharp and Dohme, AstraZeneca, COR2ED, Guardant Health, and Roche (CH); has research collaborations (nonfinancial support) with Guardant Health; advisory board activity: Archer, Inivata, Boston pharmaceutical, EMD Serono, Novartis, Pfizer, Mirati, Eisai, Daiichi Sankyo, Sanofi Genzyme-Regeneron, and BMS. Research grant from LCRF-Pfizer. The other authors declare no competing interest.

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SUPPORTING INFORMATION

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

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