Soluble PD-L1 and Serum Vascular Endothelial Growth Factor-B may Independently Predict Prognosis in Patients with Advanced Non-small Cell Lung Cancer Treated with Pembrolizumab

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Article

Soluble PD-L1 and Serum Vascular Endothelial Growth Factor-B May Independently Predict Prognosis in Patients with Advanced Non-Small Cell Lung Cancer Treated with Pembrolizumab

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Simple Summary: In this prospective cohort study, the prognostic and predictive value of baseline and post-treatment levels of serum VEGF-A, VEGF-B, sPD-1 and sPD-L1 were investigated in 55 advanced NSCLC patients treated with immune checkpoint inhibitors (ICIs). Higher pretreatment sPD-L1 and posttreatment VEGF-B levels were found to independently predict worse overall survival. VEGF-A and sPD-1 failed to show significant correlation with prognosis. None of the biomarkers was associated with treatment response. ICI-related toxicity was an independent predictor of response. VEGF-B and sPD-1 showed potential as diagnostic biomarkers, with significantly decreased levels in NSCLC patients compared to healthy controls.

Abstract: Background: A complex interplay between immune cells and abnormal tumor vasculature in the tumor microenvironment has been previously shown in preclinical studies, while clinical data suggest that angiogenesis biomarkers may be useful as predictors of prognosis and treatment response in immunotherapy-treated solid tumors, including non-small cell lung cancer (NSCLC). Our primary aim was to investigate the prognostic and predictive value of baseline and post-treatment levels of serum vascular endothelial growth factor-A and –B (VEGF-A and VEGF-B, respectively), soluble programmed cell death-1 (sPD-1) and programmed cell death-ligand 1 (sPD-L1) in patients with advanced NSCLC treated with immune checkpoint inhibitors (ICIs). **Methods:** 55 patients with advanced NSCLC eligible to receive ICIs (as monotherapy or in combination with chemotherapy) were prospectively enrolled. A group of sex- and age-matched healthy controls (n=16) was also recruited, for determination of the optimal cut-offs and the potential diagnostic value of the examined biomarkers. Serum VEGF-A, VEGF-B, sPD-1 and sPD-L1 levels were measured in peripheral blood samples using ELISA, both at baseline and at the time of treatment response evaluation, and were correlated with treatment response, prognosis (PFS, OS), and the remaining clinicopathological features of patients. **Results:** Mean age of patients was 66.5 years (SD=8.0 years); 65,5% of patients received chemotherapy and pembrolizumab combination while the remaining patients received pembrolizumab monotherapy. VEGF-B and sPD-1 levels were significantly decreased and increased, respectively, after treatment (p=0,028 and p<0,001, respectively). VEGF-A and sPD-L1 levels also decreased and increased, respectively, albeit without reaching statistical significance. ROC analysis showed that only VEGF-B and sPD-1 had significant value in discriminating between patients and controls. Univariate Cox regression analysis showed that increased pre-treatment values of sPD-1 (HR=10.96; p=0.037) and sPD-L1 (HR=1.68; p=0.040) and increased post-treatment levels of VEGF-B (HR=2.99; p=0.049) were all significantly associated with reduced OS. VEGF-B and sPD-L1 retained their prognostic significance in multivariate analysis (HR=3.37; p=0.032 and HR=2.10; p=0.014, respectively). **Conclusions:** sPD-L1 and VEGF-B may represent independent biomarkers of prognosis in advanced-stage NSCLC patients treated with ICIs.

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1. Introduction

The advent of immune checkpoint inhibitors (ICIs) has revolutionized the treatment landscape in advanced non-small cell lung cancer (NSCLC), particularly in the subset of patients with non-oncogene-addicted disease, where ICIs, with or without chemotherapy, is the recommended first-line therapy [1]. Previous trial data have shown that first-line monotherapy with single-agent ICI in this setting may significantly prolong survival as compared to platinum doublet chemotherapy, particularly among patients with high programmed death-ligand 1 (PD-L1) expression, achieving as of yet unprecedented 5-year survival rates in this selected subgroup **[2–5]**. Most, but not all, observational studies seem to confirm these results in real-world practice as well, but the maximum benefit is again observed among patients resembling the selected populations of the above pivotal trials, with regard to disease stage, performance status and PD-L1 status, while older or more fragile patients often show significantly worse outcomes **[6–9]**. Evidently, not all patients derive a significant benefit from ICIs, while those who do benefit are at risk of treatment-related toxicity due to the potential occurrence of immune-related adverse events (irAEs) of variable severity, ranging from mild to life-threatening; furthermore, secondary resistance following the initial response ensues eventually, throughout the course of treatment, in the majority of cases **[10–12]**. All the above emphasize the need for treatment stratification based on robust and longitudinal predictive biomarkers, with the ability to accurately predict which patients are more likely to respond favorably to these agents, so as to select them accordingly, thus increasing treatment efficacy while reducing the risk of unnecessary treatment-related toxicity.

Immunohistochemical PD-L1 expression, routinely assessed on histological samples as tumor proportion score (TPS), is the most widely used predictor of ICI response until now, but its real-world applications are often limited by significant challenges, mainly including the need for invasive sampling methods, its heterogeneous expression in tumor tissue, reflecting the intratumoral heterogeneity of NSCLC, as well as the suboptimal standardization of the diagnostic assays employed and variability of the scoring systems and cut-off values used [11,13,14]. Tumor mutational burden (TMB), and microsatellite instability/mismatch repair deficiency (MSI/MMR) are additional tissue-based biomarkers widely applied for prediction of ICI response, either independently or in combination with PD-L1 TPS, often providing stronger predictive information as compared to PD-L1 status alone, but failing as well to consistently provide clinically relevant and reproducible results or to accurately reflect the temporal and spatial heterogeneity of advanced NSCLC [14,15].Therefore, there clearly remains an unmet need to identify novel, ideally blood-based, predictive biomarkers in immunotherapy-treated NSCLC.

Abnormal tumor vasculature may interact with immune cells in the tumor microenvironment, in a complex interplay, ultimately leading to immune suppression which in its turn may further drive angiogenesis, “in a vicious cycle of impaired immune activation” as described by Rahma and Hodi [16]. On the other hand, antiangiogenic therapy can reverse this process, by normalizing the tumor blood vessels and restoring the normal function of immune cell factors, thus potentially enhancing the efficacy of immunotherapy as well **[17,18]**. In accordance to these preclinical data, there is increasing clinical evidence that combination of ICIs with antiangiogenic agents may not only be tolerable but also efficacious in prolonging survival as compared to ICI monotherapy in advanced NSCLC, while a variety of angiogenesis biomarkers, such as the VEGF family, are being studied for their potential utility as predictors of prognosis and treatment response in immunotherapy-treated NSCLC **[18–20]**.

The primary aim of our study was to investigate the prognostic and predictive value of baseline and post-treatment levels of a panel of angiogenesis and immune-related markers, including serum vascular endothelial growth factor-A (VEGF-A), vascular endothelial growth factor-B (VEGF-B),

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soluble programmed cell death-1 (sPD-1) and programmed cell death-ligand 1 (sPD-L1) in patients with advanced NSCLC treated with ICIs. Secondary aims included the investigation of any potential correlations between the above candidate markers and the remaining clinicopathological features studied.

2. Materials and Methods

2.1. Patient Population and Study Design

Consecutive patients (n=55) with advanced NSCLC who were eligible to receive immunotherapy (as monotherapy or in combination with chemotherapy) were prospectively enrolled. A group of sex and age-matched healthy controls (n=16) was recruited as well, for determination of the optimal cut-off point between normal and increased serum levels of VEGF-A, VEGF-B, sPD-1 and sPD-L1. The study protocol was approved by the ethics committee of our institution (approval number: 25244/25-10-19) and written informed consent was obtained from all participants prior to recruitment.

Inclusion criteria were defined as follows: written informed consent, age>18 years, histologically or cytologically confirmed diagnosis of NSCLC, advanced disease stage (IIIB to IV) and eligibility for treatment with ICIs. Patients with operable NSCLC or severe comorbidities, significantly limiting life expectancy (e.g. end-stage cardiac, renal or liver failure) or with other prior or concomitant malignancies were excluded from the study. Tumors were classified using the latest World Health Organization (WHO) histological classification. Staging was done according to the eighth edition of the International Association for Lung Cancer (IASLC) TNM classification system [21]. Standard staging procedures were used, including a complete history and physical examination, blood tests, computed tomography (CT) of the chest and abdomen, positron emission tomography (PET) scan and a computed tomography (CT) scan (PET/CT scan) and CT or magnetic resonance imaging of the brain; bone involvement was documented by PET/CT scan or bone scintigraphy.

All patients were treated with ICIs, with or without chemotherapy, as determined by the attending physician. Follow up evaluations (clinical examination, CT scan and routine laboratory investigations) were carried out at 3-month intervals. Treatment response was assessed using Response Evaluation Criteria in Solid Tumors (RECIST, version 1.1), and was classified as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD). Progression-free survival (PFS) was calculated from the start date of the first treatment cycle to the time of first documentation of PD or until death by any cause. Overall survival (OS) was defined as the time from diagnosis to the date of death by any cause.

2.2. Sample Collection

Peripheral venous blood samples (for measuring serum levels of VEGF-A, VEGF-B, sPD-1 and sPD-L1) were collected from patients at the following time points: a) at baseline/before treatment (at the first day of the first cycle of immunotherapy treatment, before administering the therapeutic agent) and b) after treatment (at the time of treatment response evaluation). All samples were allowed to coagulate at room temperature for 30 minutes. Serum was separated by centrifugation at 2000 g for 10 minutes and stored at -20°C until used for the ELISA measurements.

The levels of all biomarkers evaluated (pretreatment and posttreatment levels and the change between the two measurements) were correlated with standard clinicopathological features of patients, including age, sex, smoking history, disease stage, ECOG PS at diagnosis, histological type of tumor, immunohistochemical expression of PD-L1 in the primary tumor, type of treatment (monotherapy vs. combo), treatment-related toxicity(irAEs), treatment response, progression-free survival (PFS) and overall survival (OS).

2.3. ELISA Measurements

sPD-1, sPD-L1, VEGF-A and VEGF-B serum levels were measured in all samples in duplicate using a quantitative sandwich enzyme immunoassay technique.

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More specifically, sPD-1 was measured using the Human PD-1 sandwich ELISA Kit (ProteintechR KE00075) with intra-assay and inter-assay coefficients of variation (CV) less than 3.7% and 5.8%, respectively. The assay detection range was 125 pg/ml-8000 pg/ml. The minimum detectable dose of human PD1 is 43.0pg/ml.

sPD-L1 was measured using the Human PD-L1 sandwich ELISA Kit (ProteintechR KE00074) with intra-assay and inter-assay coefficients of variation (CV) less than 7.8% and 7.0%, respectively. The assay detection range was 0.156 ng/ml-10 ng/ml. The minimum detectable dose of human PD1 is 0.04ng/ml.

VEGF-A was measured using the Human VEGF-A ELISA Kit (InVitrogen BMS277-2) with minimum intra-assay and inter-assay coefficients of variation (CV) less than 11.7% and 8.9%, respectively. Detection range was 15.6 ng/ml-1000 pg/ml. The limit of detection was 7.9pg/ml.

VEGF-B was measured using the Human VEGF-B ELISA Kit (InVitrogen EH481RB) with intra-assay and inter-assay coefficients of variation (CV) less than 10% and CV%<12%, respectively. Detection range was 0.41 ng/ml-100 ng/ml. The minimum detectable dose of human VEGF-B was 0.4ng/ml.

2.4. Statistical Analysis

Quantitative variables were expressed as mean (Standard Deviation) or as median (interquartile range). Categorical variables were expressed as absolute and relative frequencies. For the comparison of proportions chi-square and Fisher's exact tests were used. Independent samples of Student's t-tests were used for the comparison of age between patients and healthy participants. Wilcoxon signed rank-test was used for the comparison of pre- and post- treatment biomarker values, while Spearman correlation coefficient (rho) was used to measure the strength of correlation between two quantitative variables. ROC curves (Receiver operating characteristic curves) were used in order to estimate the diagnostic ability of biomarkers. Sensitivity, specificity, negative and positive prognostic value were calculated for determination of the optimal cut-offs. The area under the curve (AUC) was also calculated. The prognostic value of each biomarker was first assessed by univariate Cox regression analysis. Variables that showed significant association with the outcome were included in the multivariate Cox proportional-hazard model in a stepwise method in order to determine the independent predictors for survival. The assumption of proportional hazards was evaluated by testing for interaction with a continuous time variable. Hazard ratios (HR) with 95% confidence intervals (95% CI) were computed from the Cox regression analyses. Kaplan – Meier survival estimates for survival were graphed over the follow-up period. All reported p values are two-tailed. Statistical significance was set at $p<0.05$ and analyses were conducted using SPSS statistical software (version 26.0).

3. Results

3.1. Clinicopathological Features of Patients

The clinicopathological characteristics of all study participants (55 patients and 16 healthy controls) are presented in table 1. Mean age of patients and controls was 66.5 years (SD=8.0 years) and 65.4 years (SD=9.1 years), respectively. Also, the majority of both patients and healthy participants were males (69.1% and 56.3% respectively). Patients and controls had similar age ($p=0.650$) and gender ($p=0.339$) distribution.

Histological type of tumor was adenocarcinoma in 35 cases (35/55, 63.6%), squamous cell carcinoma in 18 cases (18/55, 32.7%), adenosquamous carcinoma in 1 case (1/55, 1.8%) and NOS in 1 case as well (1/55, 1.8%). Most patients (51/55, 92,7%) had disease stage IV, ECOG PS 1 (34/55. 61,8%), and received first-line treatment (44/55, 84,6%). Pembrolizumab and chemotherapy combination was administered in 36/55 cases (65,5%) while the remaining patients (19/55, 34.5%) received pembrolizumab monotherapy. Disease progression was observed in 35.2% (19/55) and treatment-related toxicity (irAEs) in 40.0% (22/55) of patients.

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Table 1. Demographics and clinicopathological features of patients and controls.

	n (%)
Patients (n=55)	
Gender	
Male	38 (69.1)
Female	17 (30.9)
Age (years), mean (SD)	66.5 (8.0)
Pack-Years, mean (SD)	64.7 (27.6)
ECOG Performance Status	
0	12 (21.8)
1	34 (61.8)
2	8 (14.5)
3	1 (1.8)
Type of treatment	
Pembrolizumab monotherapy	19(34.5)
Pembrolizumab + chemotherapy	36(65.5)
Treatment line	
1	44 (84.6)
2	7 (13.5)
3	1 (1.9)
Disease Stage	
III	4 (7.3)
IV	51 (92.7)
Histological type of tumor	
Adenocarcinoma	35 (63.6)
Squamous cell carcinoma	18(32.7)
Adenosquamous carcinoma	1 (1.8)
NOS	1 (1.8)
PD-L1, mean (SD)	44.7 (36)
Response to treatment	
Partial response	14 (25.9)
Stable disease	21 (38.9)
Disease progression	19 (35.2)
Toxicity (irAEs)	22 (40.0)
Healthy controls (n=16)	
Gender	
Male	9 (56.3)
Female	7 (43.8)
Age (years), mean (SD)	65.4 (9.1)

3.2. Levels of Biomarkers and Their Diagnostic Accuracy

Pre- and post-treatment levels of the examined biomarkers are summarized in table 2. Significant changes (between pre- and post-treatment values) were found only in VEGFB (p=0,028) and sPD-1

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(p<0,001). More specifically, VEGFB decreased significantly after treatment, while sPD-1 increased significantly.

Table 2. Pretreatment and posttreatment levels of the examined biomarkers.

	n	Mean (SD)	Median (IQR)
Pre-treatment			
VEGFA	51	504.86 (311.46)	433.05 (221.39 — 731.57)
VEGFB	49	77.22 (474.79)	4.97 (3.02 — 8.2)
sPD-L1	51	0.17 (0.09)	0.16 (0.09 — 0.25)
sPD-1	51	18.17 (17.52)	12.29 (3.75 — 29.17)
Post-treatment			
VEGFA	42	430.17 (286.33)	321.19 (214.66 — 650.97)
VEGFB	42	17.69 (31.66)	6.44 (4.13 — 16.8)
sPD-L1	43	0.2 (0.11)	0.16 (0.11 — 0.3)
sPD-1	42	56.22 (99.02)	40.31 (22.92 — 52.68)
Change			
VEGFA	40	-62.95 (218.99)	-19.26 (-148.18 — 94.36)
VEGFB	38	-78.62 (514.62)	1.74 (-1.18 — 3.83)
sPD-L1	41	0.02 (0.13)	0.01 (-0.06 — 0.09)
sPD-1	40	39.97 (102.04)	19.06 (6.73 — 39.07)

The diagnostic ability of the biomarkers evaluated was examined via ROC analysis (Table 3).

Table 3. Levels of biomarkers in patients and controls and their diagnostic ability, via Roc analysis.

	Patients		Healthy sample									
	Mean (SD)	**Median (IQR)**	**Mean (SD)**	**Median (IQR)**	**ROC**	**95% CI**	**P**	**Optimal cut-off**	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
VEGFA	504.86 (311.46)	433.05 (221.39 — 731.57)	410.46 (222.32)	347.39 (256.17 — 552.22)	0.58	0.44 - 0.72	0.339	-	-	-	-	-
VEGFB	77.22 (474.79)	4.97 (3.02 — 8.2)	53.39 (39.45)	44.77 (16 — 100)	0.88	0.79 - 0.98	<0.001	≤10.94	83.7	87.5	95.3	63.6
sPD-L1	0.17 (0.09)	0.16 (0.09 — 0.25)	0.19 (0.1)	0.15 (0.12 — 0.21)	0.47	0.32 - 0.62	0.702	-	-	-	-	-
sPD-1	18.17 (17.52)	12.29 (3.75 — 29.17)	73.14 (119.28)	39.09 (25.17 — 63.39)	0.83	0.74 - 0.93	<0.001	≤34.54	80.4	62.5	87.2	50

Note. CI: Confidence Interval; PPV: Positive Prognostic value; NPV: Negative Prognostic Value. VEGFB and sPD-1 were the only markers showing a significant diagnostic value (Figures 1a and 1b).

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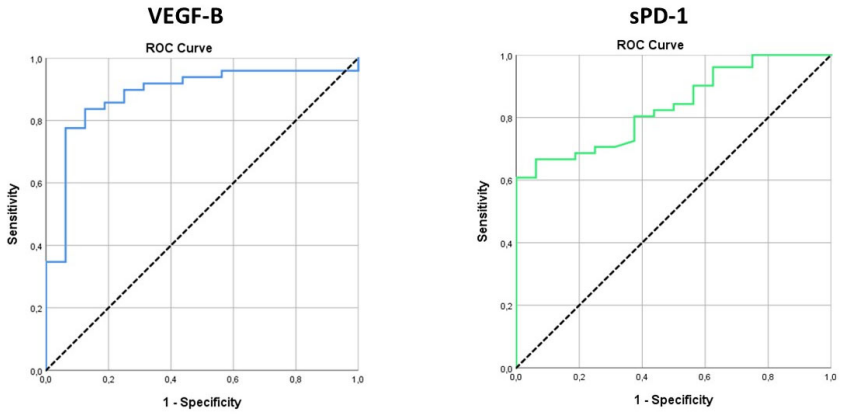

(a) (b)

Figure 1. ROC curves for a) VEGF-B and b) sPD-1.

The optimal cut-off for VEGFB was ≤10.94 pg/ml, with a sensitivity of 83.7% and specificity of 87.5%. Also, 83.7% (n=41) of the patients had VEGFB≤10.94, while the corresponding percentage for controls was 12.5% (n=2) (p<0.001). The optimal cut-off for sPD-1 was ≤34.54 pg/ml, with a sensitivity of 80.4% and a specificity of 62.5%. Also, 80.4% (n=41) of patients had sPD-1≤34.54, while the corresponding percentage for controls was 37.5% (n=6) (p=0.003).

3.3. Associations Between Biomarkers and Clinicopathological Features

Higher posttreatment VEGFA και sPD-L1 levels were associated with 2nd or 3rd line therapy. Higher posttreatment sPD-L1 levels were also correlated with the administration of pembrolizumab monotherapy.

Spearman's correlation analysis showed a significant correlation between the following variables: a) higher pack-years and greater change of sPD-L1, b) higher tumor PD-L1 (TPS) and higher post-treatment sPD-L1 και sPD-1 c)higher tumor PD-L1 and greater change of sPD-1. Spearman's correlation analysis also revealed various correlations between the study biomarkers (presented in Table 4).

Table 4. Spearman's correlation analysis results for all studied biomarkers.

		Pretreatment			Posttreatment				Change			
		VEGFB	**sPD-L1**	**sPD-1**	**VEGFA**	**VEGFB**	**sPD-L1**	**sPD-1**	**VEGFA**	**VEGFB**	**sPD-L1**	**sPD-1**
Pretreatment												
VEGFA	rho	-0,04	0,19	-0,16	0,76	0,16	0,48	0,23	-0,37	0,31	0,25	0,35
	P	0,780	0,192	0,256	**<0,001**	0,330	**0,002**	0,160	**0,019**	0,061	0,109	**0,025**
VEGFB	rho	1,00	0,26	0,09	-0,04	0,45	0,17	0,00	0,08	-0,26	-0,04	-0,05
	P		0,074	0,540	0,814	**0,005**	0,298	0,999	0,628	0,118	0,786	0,743
sPD-L1	rho		1,00	0,07	0,19	0,05	0,25	-0,12	-0,17	-0,17	-0,54	-0,10
	P			0,622	0,234	0,747	0,109	0,477	0,308	0,316	**<0,001**	0,545
sPD-1	rho			1,00	-0,36	0,38	0,18	0,32	-0,33	0,29	0,10	-0,34
	P				**0,022**	**0,014**	0,262	**0,041**	**0,040**	0,081	0,522	**0,031**
Posttreatment												
	rho				1,00	0,03	0,29	0,01	0,23	0,14	0,22	0,28

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VEGF A	P			0,851	0,067	0,974	0,155	0,393	0,172	0,085
VEGF B	rho			1,00	0,26	0,30	-0,10	0,55	0,19	0,08
	P				0,101	**0,050**	0,537	**<0,001**	0,231	0,627
sPD-L1	rho				1,00	0,43	-0,25	0,11	0,62	0,35
	P					**0,005**	0,119	0,502	**<0,001**	**0,028**
sPD-1	rho					1,00	-0,42	0,34	0,56	0,74
	P						**0,008**	**0,038**	**<0,001**	**<0,001**
Change										
VEGF A	rho						1,00	-0,20	-0,10	-0,20
	P							0,236	0,536	0,222
VEGF B	rho							1,00	0,26	0,21
	P								0,120	0,212
sPD-L1	rho								1,00	0,49
	P									**0,001**

No other statistically significant associations were observed between the biomarkers evaluated (pretreatment, post-treatment and change between the two measurements) and the remaining clinicopathological features and treatment data of patients (sex, age, ECOG PS, disease stage, treatment-related toxicity).

3.3. Correlation with Treatment Response and Survival Analysis

Mean time to disease progression was 26,9 months (SE=2,5 months). Only irAEs were found to significantly correlate with treatment response, both in univariate and in multivariate analysis; more specifically, patients who developed IrAEs had a reduction of hazard for disease progression by 87%, as compared to patients with no toxicity (HR=0.13; p=0.006).

During follow-up, 38.2% of patients (n=21) died and mean survival time was 41.2 months (SE=4.3 months) (Figure 2).

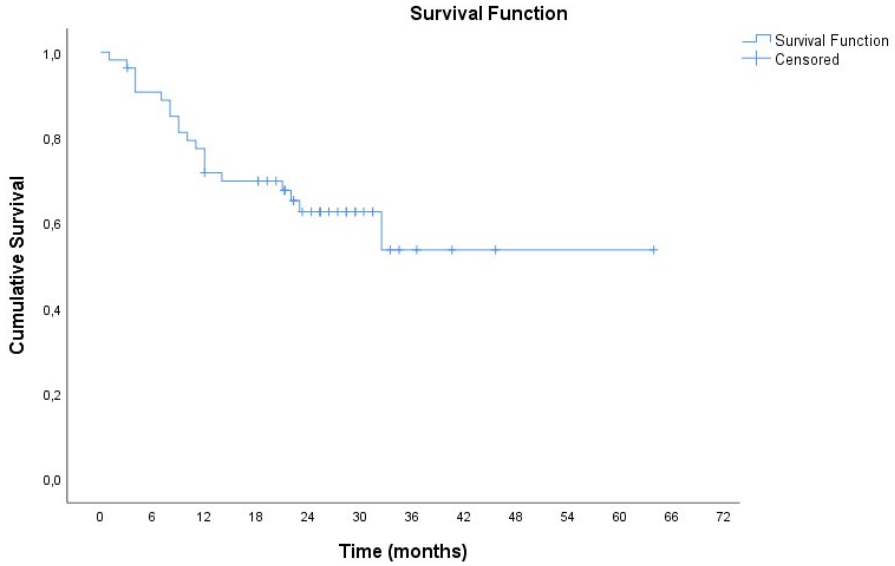

Figure 2. Kaplan-Meier curve for survival of patients.

Univariate Cox regression analysis showed that higher pre-treatment values of sPD-L1 (HR=1.68; p=0.040) and sPD-1 (HR=10.96; p=0.037) were significantly associated with greater hazard (Table 5).

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Table 5. Univariate Cox analysis results for survival, with biomarkers as independent variables.

	HR (95% CI)[1]	P
Pre-treatment		
VEGFA	0.97 (0.83 — 1.13)	0.673
VEGFB	1.03 (0.97 — 1.10)	0.297
sPD-L1	1.68 (1.02 — 2.74)	**0.040**
sPD-1	10.96 (1.15 — 104.19)	**0.037**
Post-treatment		
VEGFA	1.02 (0.85 — 1.23)	0.828
VEGFB	2.99 (1.01 — 8.89)	**0.049**
sPD-L1	0.30 (0.00 — 28.71)	0.601
sPD-1	0.90 (0.45 — 1.85)	0.777
Change		
VEGFA	0.94 (0.74 — 1.20)	0.628
VEGFB	0.96 (0.90 — 1.03)	0.243
sPD-L1	0.02 (0.00 — 1.63)	0.079
sPD-1	0.82 (0.35 — 1.96)	0.659

[1] Hazard Ratio (95% Confidence Interval).

Similarly, higher post-treatment values of VEGF-B (HR=2.99; p=0.049) were significantly associated with greater hazard. Multivariate Cox regression analysis results are presented in table 6.

Table 6. Multivariate Cox analysis results for survival in a stepwise method.

	HR (95% CI)[1]	P
sPD-L1 pre-treatment	2.10 (1,16 — 3.80)	**0.014**
VEGFB post-treatment	3.37 (1.11 — 10.22)	**0.032**

[1] Hazard Ratio (95% Confidence Interval).

Higher pre-treatment values of sPD-L1 (HR=2.10; p=0.014) and higher post-treatment values of VEGFB (HR=3.37; p=0.032) were significantly associated with greater hazard.

4. Discussion

In the present study, higher serum levels of pretreatment sPD-L1 and posttreatment VEGF-B were found to independently predict a worse OS in ICI-treated advanced stage NSCLC. The remaining biomarkers evaluated, i.e. VEGF-A and sPD-1, failed to show any statistically significant correlation with prognosis, while none of the blood-based biomarkers included in our panel was found to be significantly associated with treatment response. An independent predictive value was revealed for ICI-related toxicity only, among all parameters studied, thus confirming its well-established role as a clinical predictor of response to ICI in this setting [22].

Given the intrinsic limitations of tissue biomarkers, such as PD-L1 TPS, especially for the purpose of longitudinal real-time monitoring of patients with advanced NSCLC, there is an ongoing quest for the identification of blood-based predictors, enabling repeat evaluations throughout the disease and treatment course, without the need for invasive sampling methods. Both PD-1 and PD-L1 checkpoint molecules can be detected not only on tissue samples (as membrane-bound PD-1 and PD-L1), but in the peripheral blood as well, in the form of soluble proteins (sPD-1 and sPD-L1), thus enabling convenient monitoring of their levels at any time point needed. Most previous studies investigating baseline sPD-L1 in ICI-treated NSCLC seem to generally concur that it may represent a

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useful prognostic and predictive biomarker in this setting. A recent meta-analysis concluded that high sPD-L1 may predict a worse OS and PFS in lung cancer patients treated with ICIs [23], confirming the results of three previous meta-analyses **[24–26]**, and in agreement with the results presented herein.

Previous data on the clinical relevance of sPD-1 in ICI-treated NSCLC are much more limited (as compared to PD-L1) but seem to largely concur that higher posttreatment sPD-1 levels may represent a marker of an improved survival. As reported by Himuro et al [27], increased sPD-L1 levels at baseline were significantly correlated with worse PFS and OS in NSCLC patients receiving ICI monotherapy but not in those receiving ICIs-chemotherapy combination, while higher posttreatment sPD-1 and PD-L1 levels were predictive of an improved and worse OS, respectively, suggesting, as emphasized by the authors, that both pretreatment sPD-L1 as well as posttreatment sPD-1 and sPD-L1 may represent useful prognostic biomarkers in this setting. In another study, posttreatment sPD-1 levels were again correlated with improved OS in the ICI monotherapy subgroup of NSCLC patients [28]. Interestingly, Ohkuma et al [29] suggested a potential involvement of sPD-1 in primary resistance to anti-PD-1 ICIs in patients with various solid tumors including NSCLC, and that early changes of this marker during the course of treatment may help identify patients least likely to respond. Furthermore, a composite sPD-L1/sPD-1 biomarker for the prediction of ICI efficacy has also been proposed, based on the observed independent correlation of baseline positivity for both markers with a worse PFS [30]. In our study we failed to demonstrate any correlation between sPD-1 levels and treatment response or survival, but this may be due to inclusion of patients receiving both ICI monotherapy and ICI-chemotherapy combination.

Despite the well-established interplay between angiogenesis and immune cell factors in the tumor microenvironment **[16–18]**, exerting a critical role in the progression of NSCLC and its response to immunotherapy, research on the potential prognostic and predictive significance of peripheral blood levels of angiogenesis markers in ICI-treated NSCLC, especially with regard to VEGF-B, is sparse. In a previous study combining preclinical and clinical research, VEGF-B was shown to promote metastasis in human and mouse tumor models through remodeling of tumor microvasculature, in a process seemingly independent of VEGF-A (also known as VEGF), and despite parallel suppression of primary tumor growth; furthermore, high VEGF-B tumor tissue expression was shown to correlate with worse survival in two separate cohorts of patients with squamous cell lung cancer and melanoma, respectively, suggesting, that VEGF-B may adversely impact prognosis [31]. On the other hand, decreased tissue expression of VEGF-B (along with increased VEGF-A expression) were correlated with worse time to progression (TTP) and OS in resectable NSCLC in another study [32]. Increased levels of posttreatment VEGF-B were shown to independently correlate with reduced OS in our study, thus suggesting that VEGF-B may represent an adverse prognostic indicator in ICI-treated NSCLC. To the best of our knowledge, there is no previous study evaluating the prognostic and predictive significance of serum VEGF-B levels in this setting. Therefore, additional investigations of this candidate biomarker are warranted to further support our preliminary observations.

Previous studies on the potential prognostic and predictive relevance of VEGF-A levels in the peripheral blood of patients with ICI-treated NSCLC are limited. Continuous decrease in plasma VEGF-A levels, from baseline to day 14 of treatment, was previously correlated with prolonged PFS in patients with advanced NSCLC receiving chemotherapy-ICI combination therapy, suggesting the potential predictive value of this marker [33]. In another study, lower baseline sPD-L1 and higher post-treatment VEGF levels were both independently associated with increased and reduced PFS, respectively, in NSCLC patients treated with PD-L1 inhibitors combined with anti-angiogenetic therapy [34]. Hu et al [35] reported that increased VEGF levels at baseline was correlated with worse PFS in advanced non-small cell lung cancer treated with ICI, while Shibaki et al [36] similarly observed a worse overall response rate to anti-PD-1 antibody treatment among fragile patients with advanced NSCLC and higher serum VEGF levels, thus reinforcing the hypothesis that increased VEGF levels may be predictive of reduced efficacy to these agents.

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Although not our primary aim, in the present study we also found a potential value of VEGF-B and sPD-1 as diagnostic biomarkers. More specifically, VEGF-B and sPD-1 levels were both found to be significantly decreased in NSCLC patients as compared to healthy sex- and age-matched controls and to be able to discriminate between patients and controls with an optimal cut-off of 10.94 pg/ml and 34.54 pg/ml, respectively. These findings seem to be in contrast to some previous studies reporting increased sPD-1 levels in the serum or plasma of patients with advanced NSCLC as compared to healthy controls [37,38]. Peng et al [37] investigated the clinical significance of sPD-1 and other soluble immune checkpoint markers including sTIM-3, sCD137, sCD27, sLAG-3, sIDO, sPD-L2, sCD152 and sCD8 in NSCLC, and reported increased serum levels of all examined biomarkers (including sPD-1) in patients with advanced-stage disease versus controls; nevertheless, sPD-1 was the only marker that failed to confirm its diagnostic value in subsequent ROC analysis while a higher diagnostic accuracy was reached when a combined detection assay of sTIM-3, sLAG-3 and sCD137 was performed [37]. On the other hand, our own current findings of decreased sPD-1 levels in advanced NSCLC as compared to controls concur with those recently reported by Gu et al [39]. In the latter study, sPD-1 serum levels were found to be significantly reduced in NSCLC, and the authors hypothesized that this striking observation of a reduction instead of an increase of sPD-1 levels might be due to either reduced production of sPD-1 or to increased expression of its ligand PD-L1; increased expression of sPD-L1 may, conceivably, lead to reduction of the detectable sPD-1 levels due to binding of sPD-L1 with sPD-1 in the peripheral circulation [39].

Although strengthened by the prospective design of our study and the evaluation of all clinical aspects (diagnostic, prognostic and predictive) of the examined biomarkers, our results need to be evaluated in the context of some limitations as well. First, our patient population sample was relatively small, limiting the statistical power of our analysis and the ability to perform stratified analyses in specific subgroups, such as the pembrolizumab monotherapy group. To minimize the latter limitation, the type of ICI treatment (pembrolizumab monotherapy vs combo) was included as a variable in our study and found to be correlated with higher posttreatment sPD-L1 levels only, while failing to show a significant association with any other parameter, including of course prognosis and treatment response. An additional limitation of our study is the fact that patients receiving second-line treatment and beyond were also included, albeit as a minority subgroup (15.4%), thus reducing the homogeneity of our population but also better reflecting the characteristics of a real-world cohort. Finally, it must also be emphasized that our findings warrant additional confirmation in a validation cohort.

5. Conclusions

Our study results revealed an independent prognostic significance of pretreatment sPD-L1, thus confirming previous reports, but also highlighted serum VEGF-B, a biomarker relatively understudied as of yet, as a novel predictor of prognosis in ICI-treated advanced NSCLC. Undoubtedly, additional studies are needed to delineate the exact role of these candidate biomarkers, particularly VEGF-B, in treatment response and overall prognosis of patients. Optimization of ICI-based treatment planning in advanced NSCLC will, most likely, require designation of a combination of markers instead of a single one, following their robust validation in large prospective series.

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