Pharmacological Research 228 (2026) 108232

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Early pembrolizumab plasma levels as a prognostic biomarker in real-world NSCLC patients

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ARTICLE INFO

Keywords:
Pembrolizumab
Immunotherapy
NSCLC
Biomarker

ABSTRACT

Background: Pembrolizumab is a standard first-line treatment for metastatic non–small cell lung cancer (NSCLC). Although approved dosing regimens achieve near-maximal PD-1 receptor occupancy in clinical trials, variability in pembrolizumab exposure in real-world scenario may influence outcomes. We evaluated the prognostic relevance of pembrolizumab plasma levels after the first treatment cycle (C1 trough) in metastatic NSCLC clinical practice.

Methods: We analyzed 133 patients with metastatic NSCLC from 12 centers in Spain, who received first-line pembrolizumab as monotherapy (IO) or combined with chemotherapy (ChIO). Plasma C1 trough concentrations were measured by ELISA (High-Low cutoff; 10 μg/mL). High tumor burden was defined by SLD $\geq$ 5 cm, T-parameter $\geq$ 3, or $\geq$ 4 metastatic sites. PBMCs from 82 patients were analyzed by flow cytometry to assess free PD-1 on T cells.

Results: Low pembrolizumab C1 trough levels were significantly associated with inferior outcomes (PFS HR 2.08, $p < 0.001$; OS HR 3.06, $p < 0.001$). Female sex and fixed dosing were linked to higher exposure, largely

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https://doi.org/10.1016/j.phrs.2026.108232
Received 2 December 2025; Received in revised form 22 April 2026; Accepted 4 May 2026
Available online 5 May 2026
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explained by lower body weight and higher relative dose per kilogram. High tumor burden ($p = 0.006$) and low albumin ($r = 0.29$, $p = 0.038$) were associated to lower levels of pembrolizumab. Pembrolizumab C1 trough levels negatively correlated with PD-1 availability on CD4+ ($r = –0.422$, $p < 0.001$) and CD8+ T cells ($r = -0.250$, $p = 0.023$). In multivariate analysis, pembrolizumab C1 trough, ECOG status, and PD-L1 tumor proportion score positivity independently predicted survival.

Conclusions: Low pembrolizumab exposure after the first cycle is associated with poor survival and incomplete PD-1 blockade. C1 trough assessment may serve as a prognostic biomarker to guide individualized dosing and optimize outcomes.

1. Introduction

Non-small cell lung cancer (NSCLC) accounts for 80–85% of all lung cancer cases. NSCLC remains the leading cause of cancer-related mortality worldwide, as well as the most frequently diagnosed cancer type [1]. More than half of NSCLC patients are diagnosed at the metastatic stage, where there are no curative options and systemic treatment is the therapeutic mainstay [2]. Survival outcomes in these patients have improved remarkably over the last decade, driven by advances in staging, early diagnosis and novel therapies, especially immune checkpoint inhibitors (ICIs), such as pembrolizumab [3].

Pembrolizumab is a highly selective, humanized IgG4 monoclonal antibody that belongs to the ICI class and acts against the programmed cell death protein-1 (PD-1) [4]. The binding of programmed cell death ligand 1 (PD-L1) to its receptor PD-1 triggers inhibitory immune signaling that facilitates tumor immune evasion. The antibodies that block this interaction, reactivate the patient's immune system to attack cancer cells. ICIs have transformed the landscape of cancer treatment, showing clinical benefit across multiple tumor types.

From a pharmacokinetic perspective, pembrolizumab follows a two-compartment disposition model with predominantly linear clearance within the clinically relevant dose range. As an IgG4 monoclonal antibody, it has a relatively long half-life (22 days), and its clearance is influenced by patient-related factors such as body weight, albumin levels, and systemic inflammation. Notably, monoclonal antibody pharmacokinetics are dynamic, with increased clearance described in patients with high tumor burden or cancer-associated cachexia, likely reflecting enhanced catabolic activity and altered FcRn-mediated recycling, which may lead to lower circulating drug levels [5].

Pembrolizumab has demonstrated significant clinical benefits in metastatic NSCLC in different regimens with intravenous administration: weight-based dosing (2 mg/kg every 3 weeks, Q3W) and fixed dosing (200 mg Q3W) [4]. In addition, pembrolizumab has recently demonstrated clinical benefit when administered subcutaneously as well (790 mg every 6 weeks) [6]. However, response rates remain limited and variable, ranging from 30% to 70% [7–13]. Currently, tumor PD-L1 expression is the main predictive biomarker used in clinical practice to guide the use of pembrolizumab in first-line advanced NSCLC [10,13]. In spite of its predictive value, its measurement has both technical and clinical limitations [14].

In search of a robust predictive biomarker, interest has grown in exploring the exposure-response (E-R) relationship, through plasma quantification of ICIs and their correlation with clinical outcomes [15–19].

Data from clinical trials suggest no clear relationship between dose or exposure and clinical efficacy. In fact, the E-R curve plateaus at 2 mg/kg and above, achieving PD-1 occupancy above 95%, suggesting a maximal therapeutic effect [15–17,20]. Moreover, traditional clinicopathological characteristics, such as body weight, sex, age or tumor type, did not show a clinically significant impact on exposure [17–19].

Nevertheless, several real-world studies have shown a potential E–R relationship for pembrolizumab. For example, a prospective study in patients with metastatic melanoma found that higher pembrolizumab exposure was associated with significantly improved overall survival (OS) and progression-free survival (PFS) ($p = 0.014$ and $p = 0.045$, respectively) [21]. Similarly, a prospective study in patients with metastatic NSCLC showed shorter PFS ($p = 0.006$) and OS ($p = 0.005$) in patients in the lowest quartile of pembrolizumab plasma levels after the first dose [22].

Currently, evidence supporting the E–R relationship for anti–PD-1 therapies remains limited and inconsistent. In this context, establishing a relationship between plasma concentration and efficacy could optimize the results in terms of survival, cost-effectiveness and safety of these therapies [23,24].

The aim of this study is to assess the prognostic implications of pembrolizumab plasma concentrations after the first dose (C1 trough) in patients with metastatic NSCLC treated with first-line immunotherapy, either as monotherapy or in combination with chemotherapy, using data from the observational, multicenter, real-world study “BLI-O”.

2. Material and methods

2.1. Study cohort and design

This study included 133 patients from the BLI-O project (Ministry of Science and Innovation, grant no. RTC2019–007359–1), a multicenter initiative led by the Translational Lung Cancer Research Group at the Health Research Institute of the Puerta de Hierro Majadahonda-Segovia de Arana University Hospital (IDIPHISA).

The BLI-O project enrolled patients with metastatic NSCLC treated with standard-of-care immunotherapy (IO) or chemoimmunotherapy (ChIO) in a real-world clinical setting. All patients underwent routine molecular testing prior to treatment initiation, and those harboring known actionable oncogenic alterations (including *EGFR*, *ALK*, and *ROS1*) were excluded, in accordance with current clinical practice guidelines.

For the present analysis, 133 patients from 12 participating centers across Spain were selected based on the following inclusion criteria: (i) first-line treatment with pembrolizumab (IO or ChIO), (ii) an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, and (iii) availability of clinical follow-up and a plasma sample obtained after the first treatment cycle (C1).

All patients received pembrolizumab in accordance with European Medicines Agency (EMA)-approved regimens, either as a weight-based dose (2 mg/kg) or as a fixed dose (200 mg) Q3W.

2.2. PBMCs and plasma samples

Peripheral blood samples were collected in EDTA tubes and processed within 24 h of extraction. Samples were obtained at baseline (Blood Sample 1, BS1) and at C1, recollected the same day and prior to the administration of the second cycle of treatment (Blood Sample 2, BS2).

Plasma samples were obtained by centrifugation of whole blood at 1490 rpm for 10 min, allowing the separation of plasma from the cell fraction. Plasma was then stored at −80ºC until use.

Peripheral blood mononuclear cells (PBMCs) were isolated using the following protocol: blood was diluted 1:1 with RPMI 1640, then layered over Lymphoprep (Stemcell Technologies) for density gradient centrifugation at 1200 rpm (without brake) for 25 min. PBMCs layers were

collected, washed with RPMI 1640, and viable cells were counted using a Neubauer chamber with Trypan-Blue staining. Cryotubes containing 3–5 million cells were stored with DMSO at −180ºC until use.

2.3. Pembrolizumab levels

ELISAs targeting free pembrolizumab in plasma (pembrolizumab C1 trough levels) from BS2 extraction were performed according to manufacturer's instructions (Matriks Biotek, PEM-FD-KEY). Plasma samples were diluted 1/100 before analysis as recommended. Following the assay, absorbance was measured using a MultiSkan EX photometer (Thermo Fisher Scientific) as established by the protocol (450/650 nm).

Sample concentrations were calculated using a standard curve generated from standards provided by the kit.

2.4. Tumor burden classification

To explore the potential influence of disease extent on pembrolizumab plasma concentrations, patients were stratified according to baseline tumor burden. Tumor burden was defined using a composite criterion integrating primary tumor size, local extension, and metastatic spread. Specifically, patients were classified as having high tumor burden if they met at least one of the following conditions at baseline: (i) primary tumor size (sum of longest diameters, SLD) ≥ 5 cm as measured on baseline imaging, (ii) locally advanced disease corresponding to stage T3 or T4, or (iii) the presence of four or more distinct metastatic sites. Patients who did not fulfill any of these criteria were categorized as having low tumor burden.

2.5. Flow cytometry

PBMC samples from 83 patients in the study cohort were also analyzed by flow cytometry at BS2. Thawed PBMCs were stained using an antibody panel containing anti- CD3 (Miltenyi Biotec, 130–113–131, PerCP), anti-CD4 (Miltenyi Biotec, 130–113–230, VioGreen™), anti-CD8 (Miltenyi Biotec, 130–113–155, APC-Vio® 770), anti-CD28 (Miltenyi Biotec, 130–124–002, PE), anti-LAG3 (Miltenyi Biotec, 130–119–567, APC), anti-PD-1 (Miltenyi Biotec, 130–117–698, PE-Vio® 770) and anti-PD-L1 (BD Biosciences, 558065, FITC).

Samples were first treated and incubated for 30 min at 4ºC with FcR Blocking reagent (Miltenyi Biotec) and LIVE/DEAD TM Fixable Violet stain (Thermo Fisher) to block nonspecific binding and label dead cells. This was followed by antibody staining for 30 min at 4ºC, fixation with paraformaldehyde for 15 min at room temperature, and acquisition on a MACSQuant Analyzer 10 flow cytometer equipped with detectors for the simultaneous capture of 10 fluorescent parameters, using MACSQuantify 2.8 software (Miltenyi Biotec).

Flow cytometry data files were analyzed using FlowJoTM v10 software (FlowJo LLC). Gating strategies were performed by setting positivity thresholds based on fluorescence minus one (FMO) controls. PBMC populations were studied by their relative abundance expressed in percentage and the expression of each marker by the median fluorescence intensity (MFI).

2.6. Statistical analysis

Pembrolizumab C1 trough concentrations were categorized as High or Low using 10 μg/mL as the cut-off value. The cut-off value was based on the cohort's median pembrolizumab C1 trough concentration (9.79 μg/mL), rounded to 10 μg/mL for improved clinical applicability.

Kaplan-Meier survival analyses and log-rank tests were performed to compare these categories for PFS and OS. Bivariate correlations were assessed using Spearman's rho to evaluate associations between pembrolizumab C1 trough concentration with other quantitative variables (clinical and molecular). Chi-square or Fisher's exact tests (for sample sizes of $n < 5$) were used to analyze associations between pembrolizumab concentration categories with categorical variables. For all analyses, two-tailed p-values < 0.05 were considered statistically significant.

All statistical analyses were performed using R v4.4.2 and subsequently confirmed and graphically represented using GraphPad Prism v8.

3. Results

3.1. Cohort and sample characteristics

This study included 133 patients from the BLI-O project who met the inclusion criteria for the analysis of pembrolizumab C1 trough concentration. The median follow-up was 20.83 months (interquartile range, IQR 8.46–32.08). General patient survival analysis (**Supp. Fig. 1**) showed that at 12 months PFS was 36.67% (95% confidence interval, CI 28.14–45.22) and OS was 57.67% (95%CI 48.21–66.02). At 24 months PFS was 23.53% (95%CI 15.86–32.08) and OS was 41.79% (95%CI 32.07–51.20).

Baseline clinical characteristics of the cohort are summarized in Table 1. Patients were categorized into two groups based on their pembrolizumab C1 trough concentration: High ($\geq$10 μg/mL, $n = 64$) and Low ($<$10 μg/mL, $n = 69$).

Among all clinical characteristics, only sex and pembrolizumab dosing type showed association with pembrolizumab C1 trough concentration categories. Low pembrolizumab concentration was associated with male sex ($p = 0.008$) and weight-based dosing ($p = 0.039$) (**Supp. Fig. 2**)

3.2. High pembrolizumab C1 trough concentration is associated with better survival

Using the established cut-off value of 10 μg/mL, Kaplan-Meier survival analyses and log-rank tests were performed to assess whether pembrolizumab C1 trough concentration was associated with PFS and OS.

In the complete study cohort, high pembrolizumab C1 trough concentration was significantly associated with improved PFS ($p < 0.001$; Hazard ratio, HR=2.08, 95%CI 1.37–3.16; Fig. 1**A**) and OS ($p < 0.001$; HR=3.06, 95%CI 1.89–4.95; Fig. 1**B**). In this sense, PFS survival at 12 and 24 months were also higher for patients with high pembrolizumab C1 trough concentrations (12 m – 53.10%, 95%CI 39.87–64.68; 24 m – 34.00%, 95%CI 21.50–46.89) compared to patients with low concentrations (12 m – 20.85%, 95%CI 11.67–31.86; 24 m – 13.41%, 95%CI 5.71–24.41). Considering OS, similar results were observed with higher survivals at 12 and 24 months for high pembrolizumab C1 trough concentrations (12 m – 77.22%, 95%CI 63.91–86.13; 24 m – 61.16%, 95% CI 45.98–73.26) compared to low concentrations (12 m – 39.02%, 95% CI 26.75–51.08; 24 m – 23.62%, 95%CI 13.10–35.90).

Subgroup analyses based on cohort characteristics (sex, smoking history, ECOG performance status, pembrolizumab dosing type, tumor histology, treatment type, and PD-L1 tumor expression) showed consistent results across most subgroups, using the 10 μg/mL cutoff (**Supp. Fig. 3–10**).

Finally, the predictive value of high pembrolizumab C1 trough levels remains valid when using different cut-off points in the full cohort, as well as in ChIO and IO patients (**Supp. Table 1**).

3.3. Female patients and patients with fixed dosing received higher pembrolizumab doses, resulting in higher C1 trough concentrations

Considering the associations found between pembrolizumab C1 trough concentrations and some clinical characteristics (sex and dosing type), we performed a more detailed analysis. Firstly, survival analysis showed no associations between PFS or OS with either sex or dosing type (**Supp. Fig. 11**).

Table 1
Baseline clinical characteristics of the patient cohort. χ^2 – Chi Square Test, UMW – U-Mann Whitney Test, SD – Standard Deviation, BMI – Body Mass Index, BSA – Body Surface Area.

Characteristics	All Patients (n = 133) n (%)	High Pembrolizumab ≥ 10μg/mL (n = 64)	Low Pembrolizumab < 10μg/mL (n = 69)	p-value (Statistical Test)
Age, mean (SD)	66.09 (8.63)	66.98 (8.11)	65.26 (9.07)	0.223 (UMW)
Sex				**0.008**** (χ^2)
Female	33 (24.81)	23 (35.94)	10 (14.49)	
Male	100 (75.19)	41 (64.06)	59 (85.51)	
Smoking History				0.107 (Fisher)
Never Smoker	6 (4.51)	4 (6.25)	2 (2.90)	
Former Smoker	61 (45.87)	34 (53.13)	27 (39.13)	
Smoker	66 (49.62)	26 (40.62)	40 (57.97)	
ECOG Performance Status				1.000 (χ^2)
0	40 (30.08)	19 (29.69)	21 (30.43)	
1	93 (69.92)	45 (70.31)	48 (69.57)	
Histology				0.250 (Fisher)
Adenocarcinoma	84 (63.16)	45 (70.31)	39 (56.52)	
Squamous Cell Carcinoma	35 (26.32)	15 (23.44)	20 (28.99)	
Others	13 (9.77)	4 (6.25)	9 (13.04)	
Unknown	1 (0.75)	0 (0.00)	1 (1.45)	
Treatment				0.724 (χ^2)
Immunotherapy	53 (39.85)	27 (42.19)	26 (37.68)	
Chemoimmunotherapy	80 (60.15)	37 (57.81)	43 (62.32)	
Pembrolizumab Dosing Type				**0.039*** (χ^2)
Fixed Dose (200 mg)	52 (39.10)	32 (50.00)	20 (28.99)	
Weight-Based Dose (2 mg/kg)	62 (46.62)	25 (39.06)	37 (53.62)	
Unknown	19 (14.28)	7 (10.94)	12 (17.39)	
PD-L1				0.562 (χ^2)
Positive (≥1%)	90 (67.67)	47 (73.44)	43 (62.32)	
Negative (0%)	38 (28.57)	17 (26.56)	21 (30.43)	
Unknown	5 (3.76)	0 (0.00)	5 (7.25)	
Weight, mean (SD)	70.04 (13.84)	68.97 (13.28)	71.09 (14.41)	0.387 (UMW)
BMI, mean (SD)	25.05 (4.61)	24.79 (3.92)	25.28 (5.20)	0.717 (UMW)
BSA, mean (SD)	1.80 (0.20)	1.78 (0.20)	1.81 (0.20)	0.379 (UMW)

Focusing on sex differences, we confirmed our previous findings using the Mann-Whitney *U* test, which demonstrated that female patients (median C1 trough, 11.98 μg/mL; IQR, 9.31–14.94) had higher pembrolizumab C1 trough concentrations than male patients (median C1 trough, 9.01 μg/mL; IQR, 6.80–12.18) ($p = 0.001$, Fig. 2**A**). However, when stratifying the results by dosing type, these differences were significant only in the fixed dose group ($p = 0.003$). Female patients had significantly lower body weight compared with male patients ($p < 0.001$), resulting in female patients receiving a higher pembrolizumab dose per kilogram ($p = 0.017$, Fig. 2**B**).

On the other hand, focusing on differences between dosing types, we confirmed using the Mann-Whitney *U* test that patients with fixed dosing had higher pembrolizumab C1 trough concentrations (median C1 trough, 12.21 μg/mL; IQR, 6.97–14.74) than those with weight-based dosing (median C1 trough, 9.09 μg/mL; IQR, 6.92–11.30) ($p = 0.004$, Fig. 2**C**). Moreover, patients with fixed dosing received a higher pembrolizumab dose per kilogram (median dose, 2.94; IQR, 2.47–3.3 mg/kg) compared with patients with weight-based dosing, who all received 2 mg/kg ($p < 0.001$, Fig. 2**D**).

Finally, we observed that pembrolizumab dose per kilogram was positively correlated with C1 trough concentration (Pearson's $r = 0.403$, $p = 0.003$, Fig. 2**E**).

3.4. Baseline albumin and tumor burden are associated to pembrolizumab C1 trough concentration

We next assessed whether pembrolizumab plasma concentrations could be influenced by patient catabolic status and tumor burden.

Baseline biochemistry data were available for a subset of patients (n = 57), allowing us to explore potential correlations between blood metabolic parameters and pembrolizumab C1 trough concentration. Among the variables analyzed, only albumin showed a slight but significant positive correlation with pembrolizumab C1 trough concentrations (Pearson's $r = 0.291$, $p = 0.038$, Fig. 3**A**). No significant correlations were observed with the remaining metabolic parameters (Fig. 3**B and Supp. Table 2**).

Patients with high tumor burden (n = 32; median C1 trough, 7.87 μg/mL; IQR, 7.25–9.54) exhibited significantly lower pembrolizumab C1 trough concentrations than those with low tumor burden (n = 19; median C1 trough, 10.98 μg/mL; IQR, 9.26–13.00; $p = 0.007$) (Fig. 3**C, left panel**). This association remained consistent when patients were categorized according to pembrolizumab levels using 10 μg/mL: 75% of patients with high tumor burden had low pembrolizumab concentrations compared with only 37% of those with low tumor burden (Chi-square test, $p = 0.007$; Fig. 3**C, right panel**).

Notably, no significant differences in progression-free survival were observed between patients with high and low tumor burden ($p = 0.589$; HR=1.23; 95% CI, 0.59–2.60), although a significant association was observed for overall survival ($p = 0.031$; HR=3.01; 95% CI, 1.24–7.30) (**Supp. Fig. 12**).

Finally, no significant differences in baseline albumin levels were observed between the high and low tumor burden groups (Fig. 3**D**).

3.5. Pembrolizumab C1 trough concentrations negatively correlates with PD-1 availability on T cell populations

In addition to baseline biochemistry data, a subset of the pembrolizumab levels study cohort also had their PBMCs analyzed by flow cytometry (n = 82 / 61.65%) at the same timepoint as the measurement of pembrolizumab concentration (C1).

Given that pembrolizumab binds to PD-1, we focused our analysis on PD-1 expressing T lymphocytes. Importantly, the anti-PD-1 antibody

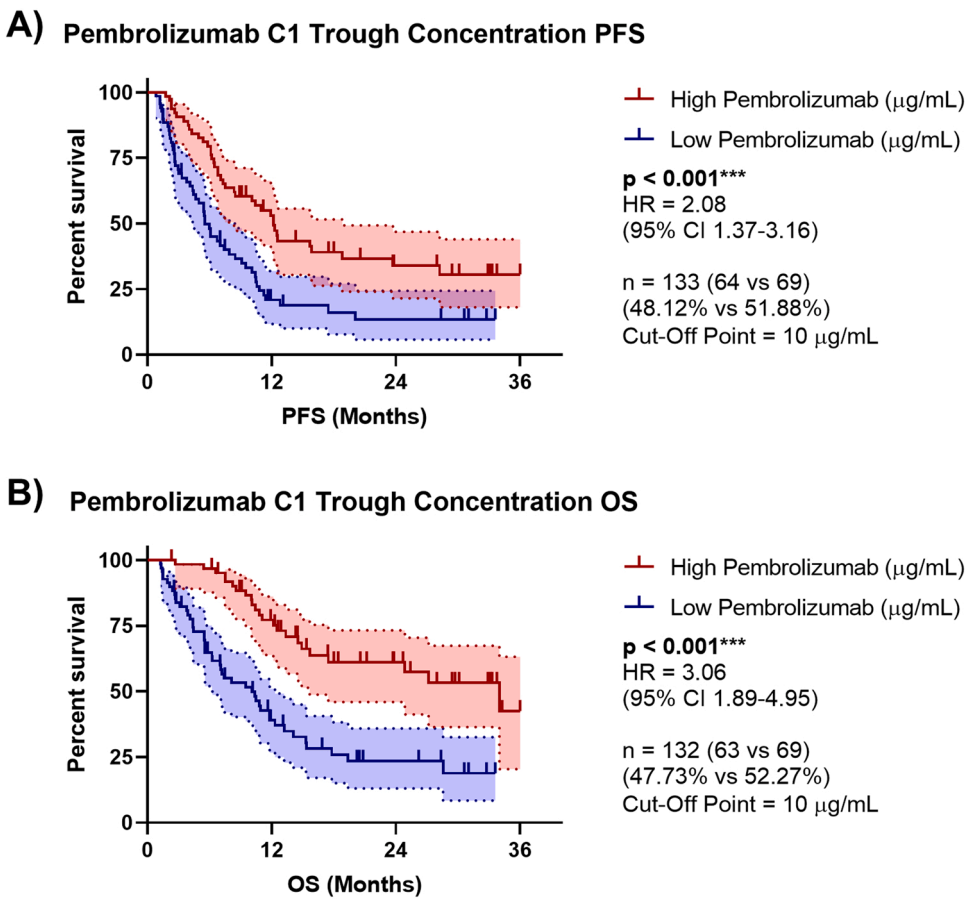


Fig. 1. Pembrolizumab C1 trough concentration and survival outcomes. (A) Patients with high pembrolizumab C1 trough concentrations (≥ 10 μg/mL) had longer PFS with a median 12.17 months, compared to 5.57 months for those with low concentrations. **(B)** Patients with high pembrolizumab C1 trough concentrations (≥ 10 μg/mL) had longer OS with a median of 34.00 months compared to 10.10 months for patients with low concentrations. HR – Hazard ratio.

used for flow cytometry competes with pembrolizumab for PD-1 binding; therefore, PD-1 staining during treatment reflects the amount of free (unbound) PD-1 protein on the T-cell surface.

Specifically, we assessed correlations between pembrolizumab C1 trough concentration and two readouts: the detection of PD-1+ T-cells (as frequency of cells) and the PD-1 protein expression (as median fluorescence intensity, MFI of the antibody).

The PD-1 MFI in both CD4+ and CD8+ PD-1+ T lymphocytes in blood was negatively correlated with pembrolizumab C1 trough concentration (CD4+: Pearson's r = -0.422, $p < 0.001$; CD8+: Pearson's r = -0.250, $p = 0.023$) (Fig. 4**A-B**). In addition, the frequency of CD4+ PD-1+ T lymphocytes was also negatively correlated with pembrolizumab C1 trough concentration (Pearson's r = -0.283, $p = 0.010$) (Fig. 4**C**). In contrast, the frequency of CD8+ PD-1+ T lymphocytes did not show any significant correlation with pembrolizumab trough concentrations (Pearson's r = -0.086, $p = 0.440$) (Fig. 4**D**).

3.6. Pembrolizumab C1 Trough Concentration is an Independent Prognostic Biomarker

To assess whether pembrolizumab C1 trough concentration independently predicted clinical outcomes, we performed univariate and multivariate Cox regression analyses for both PFS and OS.

In the univariate analyses, variables significantly associated with PFS were ECOG performance status, PD-L1 tumor proportion score (TPS) positivity, and pembrolizumab C1 trough concentration. For OS, significant variables were ECOG performance status, tumor histology, tumor burden, and pembrolizumab C1 trough concentration (**Supp. Fig. 13**). Notably, low pembrolizumab C1 trough concentration exhibited the strongest association with poor outcomes compared to other variables, with hazard ratios of 2.12 (95% CI, 1.39–3.22; $p < 0.001$) for disease progression and 3.18 (95% CI, 1.91–5.30; $p < 0.001$) for death.

Variables showing a significant association in the univariate analysis were subsequently included in the multivariate Cox models. For PFS, ECOG performance status (HR = 2.06; 95% CI, 1.23–3.46; $p = 0.006$), PD-L1 TPS positivity (HR = 1.90; 95% CI, 1.19–3.04; $p = 0.007$), and pembrolizumab C1 trough concentration (HR = 2.11; 95% CI, 1.38–3.23; $p = 0.001$) all remained independently associated with progression risk (n = 128; Fig. 5**A**).

For OS, pembrolizumab C1 trough concentration was the only variable that retained independent prognostic significance in the multivariate model with less patients available for this analysis (HR = 4.45; 95% CI, 1.33–14.94; $p = 0.016$; n = 41; Fig. 5**B**).

4. Discussion

In this study, we examined plasma concentrations of pembrolizumab to explore the relationship between pembrolizumab exposure and survival outcomes in patients with metastatic NSCLC treated with immunotherapy. The C1 trough concentration was selected to minimize potential confounding effects from treatment-related factors, such as chemotherapy-induced tissue damage or early tumor responses. This early timepoint enables a more accurate assessment of intrinsic

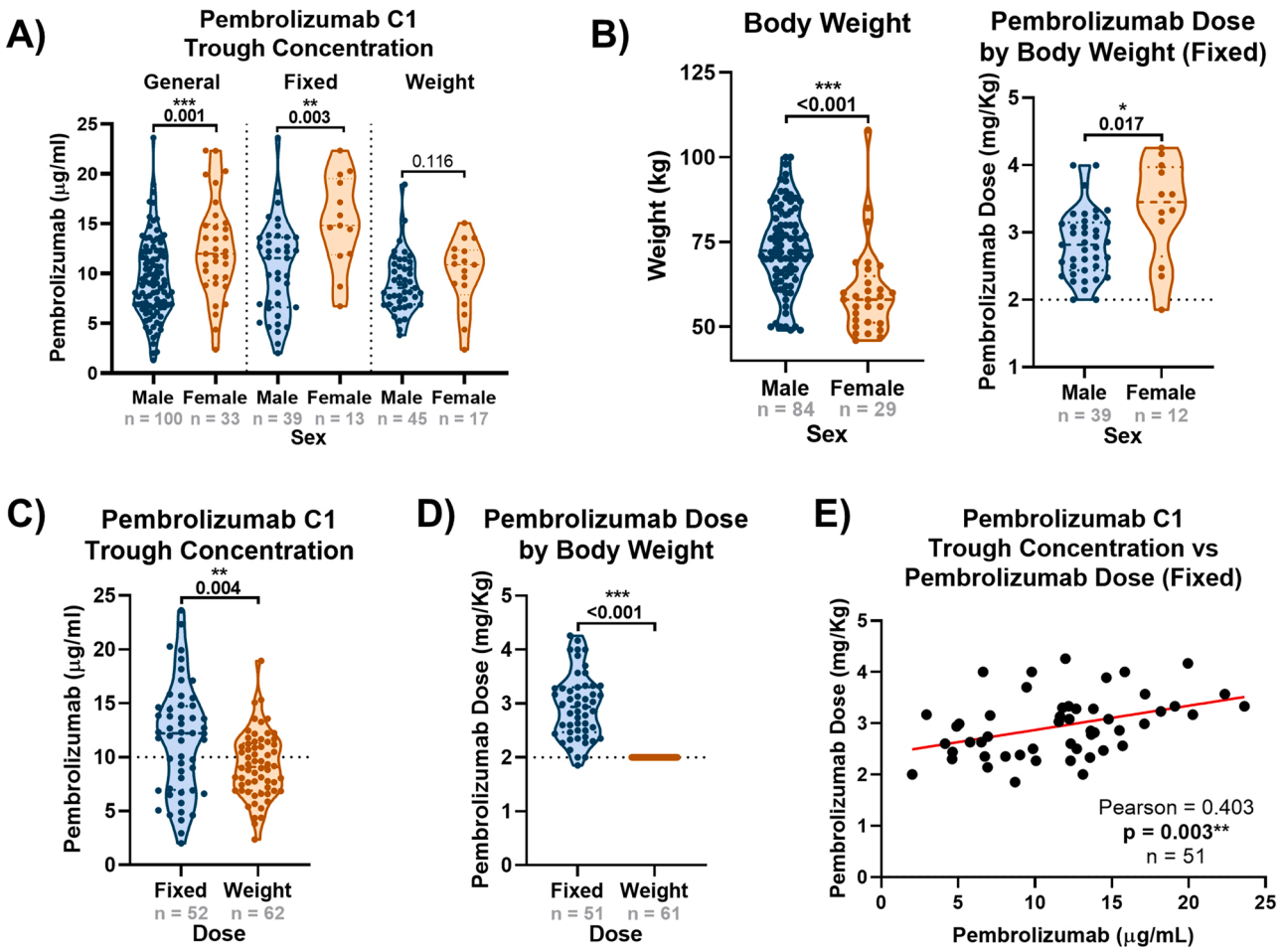


Fig. 2. Females and fixed dosed patients had higher pembrolizumab C1 trough concentrations due to a higher dose per body weight. (A) Female patients receiving a fixed dose had higher pembrolizumab C1 trough concentrations. **(B)** Female patients had lower body weight than male patients, resulting in a higher dose per kilogram under fixed dosing. **(C)** Patients with fixed dosing had higher pembrolizumab C1 trough concentrations compared to patients with weight-based dosing. **(D)** Patients with fixed dosing received a higher pembrolizumab dose per kilogram. **(E)** Pembrolizumab C1 trough concentration was positively correlated to pembrolizumab dose per kilogram. The 10 µg/mL threshold used in this study, as well as the standard clinical dose of 2 mg/kg, are indicated as horizontal lines for reference.

pharmacokinetic variability and provides an opportunity to evaluate pembrolizumab exposure as an early biomarker to inform clinical decision-making.

In our population, plasma levels of pembrolizumab after the first cycle greater than 10 µg/mL were significantly associated with improved PFS and OS. Similar associations were observed across alternative exposure thresholds, supporting the robustness of this biomarker. This finding is consistent with an E-R relationship, particularly in the context of real-world clinical practice.

Our results are discordant with available evidence from clinical trials using pembrolizumab. In these trials, the E-R curve plateaued at a dose of 2 mg/kg Q3W [25], achieving at least 95% target engagement. This suggests a maximal therapeutic effect with a flat E-R relationship across the clinical dose range [20]. However, real-world evidence supports the notion that pembrolizumab exposure may have greater variability and potential clinical relevance in non-selected population.

Our work, as well as that of Yagishita et.al., reported an association between C1 trough concentration and survival outcomes. In their cohort of NSCLC patients over 75 years of age, those in the lowest quartile of C1 trough (2.24–9.23 µg/mL) experienced poorer PFS (3.4 vs. 11.8 months, $p = 0.006$) and OS (9.9 vs. 21.7 months, $p = 0.005$) than in other quartiles [22]. Beyond NSCLC, other studies have demonstrated a significant E-R relationship with anti-PD1 in melanoma [21,26]. Thus, Navani et.al. [21], showed that patients with higher pembrolizumab plasma concentrations experienced longer OS and PFS, and had improved overall response. Similarly, Mallardo et al. reported that higher serum concentrations of nivolumab 12 weeks after treatment start (>32 µg/mL) were associated with better PFS and OS outcomes in patients with metastatic melanoma [26].

Regarding the determinants of variability in pembrolizumab levels, our study identified differences according to sex and dosing regimen, with lower drug exposure observed in male patients and in those receiving weight-based dosing. However, these differences seemed to be explained by confounding factors related to body weight and its impact on the relative dose (mg/kg). Female patients had lower average body weight and consequently may have received a higher relative dose. Similarly, higher relative dose was also observed with the fixed-dose regimen, resulting in greater drug exposure. Nonetheless, no impact of sex or dosage type on survival (PFS or OS) was found, as has been demonstrated in previous studies [27,28].

Nevertheless, our findings also suggest that systemic exposure to pembrolizumab varies according to the dosing strategy, which appears to differ from previously published modeling and simulation data supporting the equivalence between fixed dosing of 200 mg and weight-based dosing of 2 mg/kg [19,29].

Beyond body weight and pembrolizumab dose as possible determinants of drug exposure in real-world settings, our study also identified an association between pembrolizumab plasma

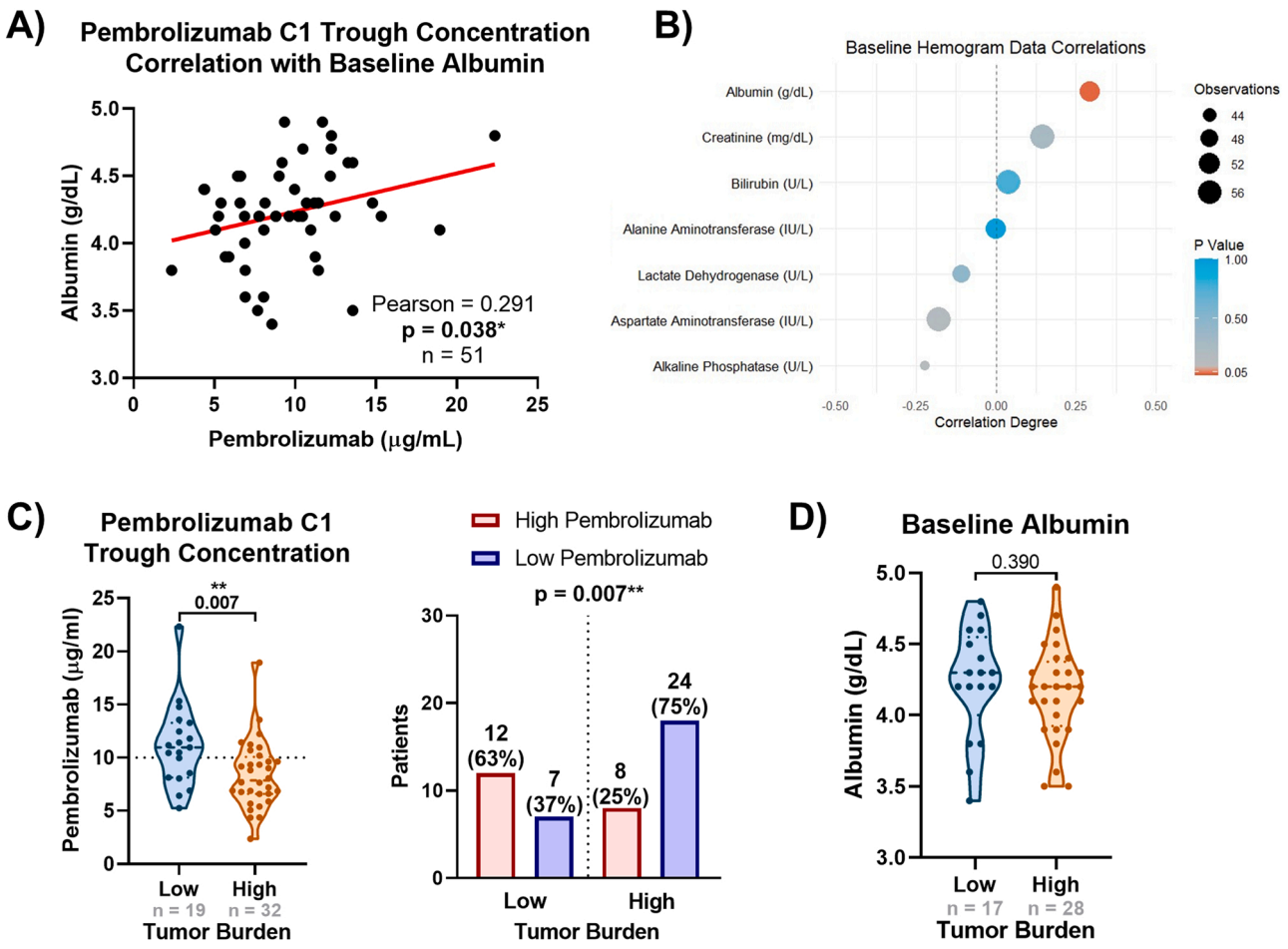


Fig. 3. Correlation between hematologic data and pembrolizumab C1 trough concentration. A) Positive correlation of pembrolizumab C1 trough concentration with baseline albumin. **B)** Correlation results of all metabolic parameters with pembrolizumab C1 trough concentration. **C)** Pembrolizumab C1 trough levels in patients with high or low tumor burden. **D)** Albumin levels in patients with high or low tumor burden. The 10 μg/mL threshold used in this study is indicated as a horizontal line for reference.

concentrations and baseline albumin levels or tumor burden.

In this context, low albumin levels in patients with otherwise preserved nutritional status could reflect reduced neonatal Fc receptor (FcRn) activity, leading to increased pembrolizumab clearance. FcRn plays a crucial role in recycling and protecting immunoglobulins from lysosomal degradation. It binds both IgG and albumin in a non-competitive manner, thereby prolonging their half-life in circulation [30–32]. Alterations in the function of this receptor, whether due to genetic variations or disease-related changes, could lead to increased clearance of pembrolizumab and, consequently, reduced exposure, which may be associated with reduced therapeutic efficacy [24,30,33].

Additionally, tumor burden and overall patient condition may contribute to systemic catabolic activity and enhanced monoclonal antibody degradation [34,35]. Consistent with this hypothesis, we observed lower pembrolizumab plasma concentrations in patients with higher baseline tumor burden, in agreement with previous studies reporting that low albumin levels, increased systemic catabolism, and high disease burden are associated with enhanced monoclonal antibody clearance and poorer outcomes in terms of PFS and OS [36–39]. However, in those reports, the relationship between high clearance and poor prognosis was interpreted uniquely as a reflection of the patient's poor general condition completely discarding the detrimental effect of low drug levels and suggesting that dose escalation would not overcome the unfavorable prognosis.

In contrast, our findings appear to support both mechanisms, the notion that pembrolizumab levels may act as an indirect biomarker of tumor burden, while also being associated with therapeutic efficacy.

Although a direct causal relationship between low drug exposure and disease progression cannot be definitively established, our findings support a potential impact on therapeutic efficacy. Specifically, we observed a negative correlation between pembrolizumab plasma levels and detectable PD-1 expression on circulating T cells, suggesting that suboptimal receptor blockade may occur in patients with lower drug exposure. This observation reinforces a biologically plausible link between drug exposure and target engagement.

Such incomplete PD-1 inhibition could allow persistent PD-L1/PD-L2 interaction, promoting immune evasion and disease progression. Thus, free PD-1 on lymphocytes may represent a complementary pharmacodynamic biomarker to plasma pembrolizumab concentrations. To our knowledge, this is one of the first studies to explore the relationship between plasma concentrations of pembrolizumab and the functional bioavailability of PD-1 in immune subpopulations, which may allow future studies integrating pharmacokinetics and pharmacodynamics. Although pembrolizumab plasma levels in our cohort may be partially influenced by tumor burden, this factor alone does not appear to fully explain the prognostic value of drug exposure on disease progression or death. Indeed, tumor burden itself was not associated with PFS in our study, and its predictive value for OS was lost after multivariate adjustment.

Notably, pembrolizumab C1 trough concentration showed potential to be an independent prognostic factor for both PFS and OS in multivariate analyses, indicating that pembrolizumab exposure may provide

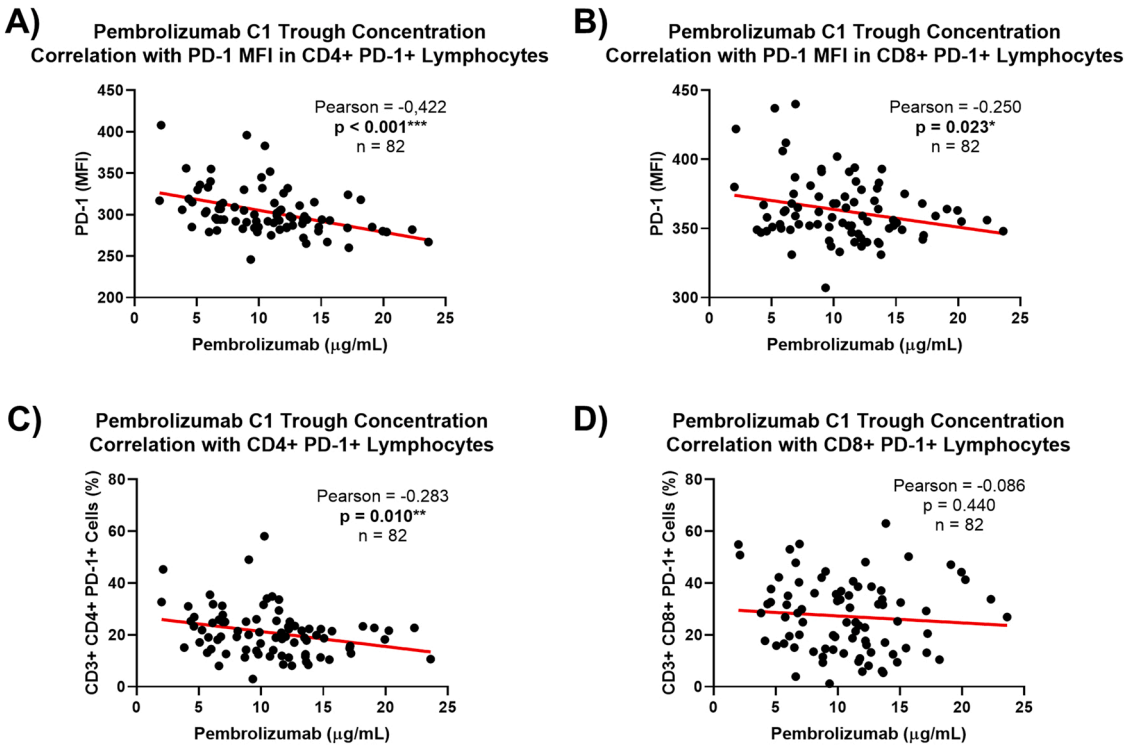


Fig. 4. Negative correlations between PD-1+ T cell populations and pembrolizumab C1 trough concentration. Correlations of pembrolizumab C1 trough concentration with, **(A)** PD-1 MFI in CD4+ PD-1+ T lymphocytes and **(B)** CD8+ PD-1+ T lymphocytes, and with **(C)** the frequency of CD4+ PD-1+ T lymphocyte detection and **(D)** CD8+ PD-1+ T lymphocytes.

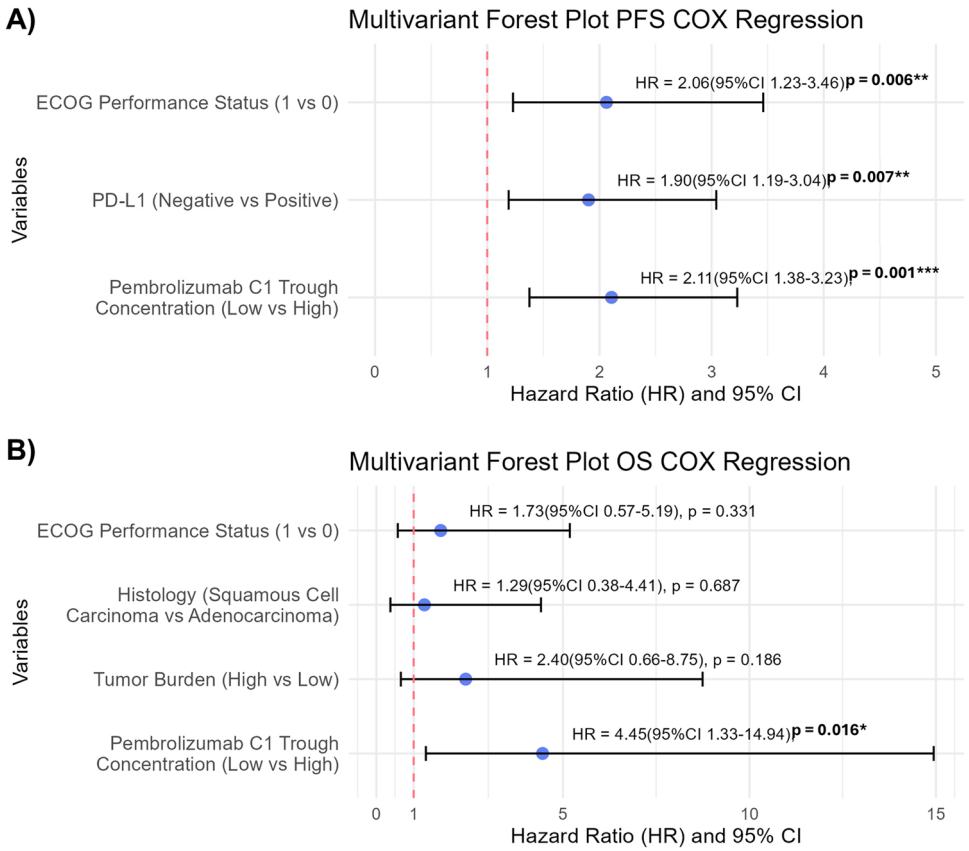


Fig. 5. Forest plots illustrating the results of the multivariate Cox proportional hazards analysis in the patient cohort, showing the hazard ratios and 95% confidence intervals for progression-free survival (PFS) **(A)** and overall survival (OS) **(B).**

prognostic information beyond performance status and baseline disease extent.

This study has several limitations and its exploratory findings should be interpreted with caution. Its retrospective observational design may have introduced biases, and pembrolizumab levels were measured at a single post–cycle 1 time point, precluding full pharmacokinetic characterization. The relatively limited sample size also constrained the statistical power of subgroup analyses—particularly in smaller subgroups such as female patients, those with squamous histology, or patients treated with pembrolizumab monotherapy—where consistent trends were observed but did not reach statistical significance. Data on FcRn function or polymorphisms were not available, warranting further studies to clarify the mechanisms underlying pembrolizumab exposure variability. Finally, the absence of an external validation cohort limits the generalizability of the results. Nonetheless, this simple approach may provide valuable early prognostic information, and the multicenter design together with consistent outcomes across subgroups support the robustness and clinical relevance of the study.

5. Conclusions

Our findings suggest that assessing pembrolizumab C1 trough concentrations may represent a promising early biomarker tool to identify metastatic NSCLC patients less likely to benefit from immunotherapy. Moreover, associations between plasma drug levels with higher administered dose, lower body weight, higher albumin, lower disease burden, and lower free PD-1 on lymphocyte subsets support the development of predictive models to estimate drug exposure. The existence of a relationship between actual drug exposure and its efficacy could inform pharmacodynamic monitoring and dose optimization strategies, pending prospective validation, potentially contributing to a more personalized use of immunotherapy.

Ethics approval and consent to participate

Sample collection and processing followed applicable ethical regulations for human biological specimens, including the Deontological Code and the Declaration of Helsinki. The study protocol was approved by the Clinical Research Ethics Committee of Puerta de Hierro University Hospital (ref. 15.20), and all participants provided written informed consent for the use of their biological samples in the study.

Funding

Work in the authors' laboratories was supported by "Instituto de Salud Carlos III" (ISCIII) PI22/01223 grant cofounded by European Regional Development Fund (ERDF), Ministry of Science and Innovation RTC2019–007359–1 'BLI-O', CPP2022–009545 'STRAGEN-IO', and European Union's Horizon 2020 research and innovation programme, P4-LUCAT project (PERME19002RODR and AC19/00026), to MP. AL-M received a research project grant funded by 'XVI convocatoria de ayudas para el desarrollo de proyectos de Investigación Fundación Universidad Alfonso X El Sabio - Santander Universidades 2023'. AC-B is supported by a "Miguel Servet" contract CP23/00044 by ISCIII and received an ISCIII project grant PI23/01054 both cofounded by European Union. CM-T is supported by Comunidad de Madrid PIPF-2022/SAL-GL-25283 contract granted to MP. MM-A is supported by Ayuda Predoctoral Asociación Española Contra el Cáncer (AECC) Madrid 2023 contract granted to MP. AnG-G was supported by Comunidad de Madrid and European social fund PEJ-2023-AI/SAL-GL-27634 contract, and now is supported by FI24/00270 predoctoral contract from ISCIII granted to AC-B. JG-E supported by Comunidad de Madrid and European social fund PEJ-2023-AI/SAL-GL- 27634 contract granted to AC-B.

The study sponsors had no role in the design of the study, the collection, analysis, or interpretation of data, the writing of the manuscript, or the decision to submit the paper for publication.

CRediT authorship contribution statement

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Patient consent for publication

Not applicable.

Declaration of Competing Interest

MJ-M declares Payment or honoraria for lectures, presentations or speakers bureaus from Roche, Lilly, Novartis and Janssen; Support for attending meetings and/or travel from Lilly and Janssen.

VC reports consulting fees from Roche, Astra Zeneca, MSD, BMS, Takeda, Sanofi, Amgen, GSK, Boehringer Ingelheim and Janssen; honoraria for lectures from Roche, Astra Zeneca, MSD, BMS, Takeda, Sanofi, Amgen, Pfizer, Janssen and Beigene; and support for attending meetings from Astra Zeneca, Roche, MSD, Takeda and Janssen.

AC-L reports consulting fees from Pfizer; honoraria for lectures from Astra Zeneca, Regeneron, Takeda; and support for attending meetings from Pfizer, Takeda, BMS, Roche, MSD and Janssen.

PD-T declares Payment or honoraria for lectures, presentations or speakers bureaus from BRISTOL-MYERS SQUIBB, Astra-Zeneca, Roche, MSD, Takeda, Pfizer and Amgen; sup- port for attending meetings and/or travel from BRISTOL-MYERS SQUIBB, Roche, MSD and Takeda; Participation on a Data Safety Monitoring Board or Advisory Board from BRISTOL-MYERS SQUIBB, Astra-Zeneca, and Boehringer Ingelheim; and other financial or non- financial interests from Astra-Zeneca, Roche, Mirati, and Pfizer.

PD-T reports grants from Foundation of SLCG; payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from BMS, Astra Zeneca, Roche, MSD, Takeda, Pfizer and Amgen; support for attending meetings and/or travel from BMS, Roche, MSD and Takeda; participation on a data safety monitoring board or advisory board from BMS, Astra Zeneca and Boehringer Ingelheim; and other financial or non-financial interests from Astra-Zeneca, Roche, Mirati and Pfizer.

AL-M declares Payment or honoraria for lectures or presentations from Astra Zeneca, BMS, Roche and Lilly; Support for attending meetings and/or travel from MSD, BMS, Pierre Fabre, Roche and Takeda.

JR declares speaker or advisory fees for Roche, Astra ZENECA, Merck, MSD, BMS, and travel expenses from BMS, MSD, ROCHE, Merck, MSD.

AS-G reports receiving payments or honoraria for lectures, presentations, participation in speakers' bureaus, manuscript writing, or educational events from AstraZeneca, Pfizer, Deciphera, and Roche Farma. Support for attending meetings and/or travel from Deciphera, Roche Farma, Pfizer, AstraZeneca, and PharmaMar; and has also participated on Data Safety Monitoring Boards or Advisory Boards for Roche Farma and Boehringer Ingelheim.

AS-H declares Payment or honoraria for lectures, presentations or speakers bureaus from Roche, Lilly, MSD, Sanofi, Astra Zeneca, Janssen, Takeda, Merck, Novartis, Pfizer, and Bristol- Myers Squibb; Support for attending meetings and/or travel from Roche, Sanofi, MSD Takeda, Janssen, Pfizer, and Bristol-Myers Squibb. AE declares Payment or honoraria for lectures, presentations or speakers bureaus from MSD, Pharmamar, Astra Zeneca, Takeda, Roche, and GSK; Support for attending meetings and/or travel from MSD, Boehringer Ingelheim, and Pharmamar; Participation on a Data Safety Monitoring Board or Advisory Board from Roche and MSD.

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MP reports grants, consulting fees and non-financial support from Bristol Myers Squibb, Roche, and AstraZeneca; and consulting fees from MSD and Takeda. All other authors declare no competing interests.

The rest of authors declare no potential conflicts of interest.

Acknowledgements

We thank the patients and their families and the clinical teams and the Spanish Lung Cancer Group. We thank technicians Ana Isabel Rodríguez, Daniel Lobato, and Sergio Espartero (Translational lung cancer research group, department of Medical Oncology, IDIPHISA) for excellent technical assistance.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2026.108232.

Data availability

Data will be made available on request.

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