

Clinical potential of circulating free DNA and circulating tumour cells in patients with metastatic non-small-cell lung cancer treated with pembrolizumab

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Keywords

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Immune checkpoint inhibitors, such as pembrolizumab, are revolutionizing therapeutic strategies for different cancer types, including non-small-cell lung cancer (NSCLC). However, only a subset of patients benefits from this therapy, and new biomarkers are needed to select better candidates. In this study, we explored the value of liquid biopsy analyses, including circulating free DNA (cfDNA) and circulating tumour cells (CTCs), as a prognostic or predictive tool to guide pembrolizumab therapy. For this purpose, a total of 109 blood samples were collected from 50 patients with advanced NSCLC prior to treatment onset and at 6 and 12 weeks after the initiation of pembrolizumab. Plasma cfDNA was measured using *hTERT* quantitative PCR assay. The CTC levels at baseline were also analysed using two enrichment technologies (CellSearch[®] and Parsortix systems) to evaluate the efficacy of both approaches at detecting the presence of programmed cell death ligand 1 on CTCs. Notably, patients with high baseline *hTERT* cfDNA levels had significantly shorter progression-free survival (PFS) and overall survival (OS) than those with low baseline levels. Moreover, patients with unfavourable changes in the *hTERT* cfDNA levels from baseline to 12 weeks showed a higher risk of disease progression. Additionally, patients in whom CTCs were

Abbreviations

APC, allophycocyanin; AUC, area under the curve; cfDNA, circulating free DNA; CKs, cytokeratins; Cq, quantification values; CR, complete response; CTCs, circulating tumour cells; DAPI, dye 4',6-diamidino-2-phenylindole; ECOG PS, Eastern Cooperative Oncology Group Performance Status; FDA, Food and Drug Administration; FLU, fluorescein; ICIs, immune checkpoint inhibitors; IHC, immunohistochemistry; NSCLC, non-small-cell lung cancer; ORR, objective response rate; OS, overall survival; PD, progressive disease; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; PE, phycoerythrin; PFS, progression-free survival; PR, partial response; qPCR, quantitative PCR; ROC, receiver operating characteristic; SD, stable disease; TMB, tumour mutational burden; TPS, tumour proportion score.

detected using the CellSearch® system had significantly shorter PFS and OS than patients who had no CTCs. Finally, multivariate regression analyses confirmed the value of the combination of CTCs and cfDNA levels as an early independent predictor of disease progression, identifying a subgroup of patients who were negative for CTCs, who presented low levels of cfDNA and who particularly benefited from the treatment.

1. Introduction

Lung cancer is the most commonly diagnosed cancer, ranking first in morbidity and mortality rates among malignant tumours worldwide [1]. Under normal physiological conditions, immune checkpoint proteins are crucial for the maintenance of self-tolerance, protecting tissues from damage when the immune system responds to infections. However, the expression of immune checkpoint proteins is dysregulated by tumours as an immune resistance mechanism [2]. Over the past decade, immunotherapy has become a milestone in the treatment of non-small-cell lung cancer (NSCLC), which accounts for 80–90% of all lung cancer cases [3].

Currently, immune checkpoint inhibitors (ICIs) target both programmed cell death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1). Pembrolizumab is a humanized IgG4 monoclonal antibody that inhibits the PD-1 receptor. When used as a monotherapy, it is the standard first-line treatment for selected patients with metastatic NSCLC presenting high PD-L1 tissue expression ($\geq 50\%$) [4]. The standard method to determine the levels of PD-L1 is immunohistochemistry (IHC) of tumour tissues, which is recommended for all patients with newly diagnosed advanced NSCLC in routine clinical practice [5]. However, PD-L1 expression does not seem to be an optimal predictive biomarker since not all patients experience an effective response to ICIs based on the established selection criteria [6]. Thus, the addition of pembrolizumab to platinum-based chemotherapy in patients with previously untreated advanced NSCLC has recently produced a significant improvement in survival outcomes, independent of PD-L1 expression [7,8].

The determination of PD-L1 expression in tissues is highly variable according to the time and site of biopsy, and sometimes, PD-L1 expression is not detected due to the limited tissue sample. Moreover, a unique tissue biopsy may not be representative of the entire molecular landscape of the tumour, and therefore, some PD-L1-positive patients do not receive immunotherapy. The management of NSCLC with ICIs requires the identification of new and reliable biomarkers to select patients who will benefit from immunotherapy while limiting

ineffective therapy that may produce adverse reactions in patients [9]. Liquid biopsy has emerged as a rapid and noninvasive alternative tool to obtain new biomarkers of several cancers and to monitor its evolution over time [10–12]. Circulating free DNA (cfDNA) and circulating tumour cells (CTCs) are the most common and standardized liquid biopsy biomarkers, representing promising tools for the diagnosis, selection of ICI treatment and monitoring of patients with NSCLC receiving immunotherapy [13]. Moreover, analyses using combinations of multiple liquid biopsy biomarkers are being conducted to improve the accuracy of detection [14,15].

Some studies have suggested that monitoring cfDNA dynamics might help clinicians select patients with NSCLC who will benefit most from immunotherapy [15–17]. In this study, we explored the value of the cfDNA determination to anticipate the evolution of metastatic NSCLC in patients receiving first-line pembrolizumab as a monotherapy or combination therapy. In addition, we also focused our attention on CTC levels, including an analysis of the PD-L1-positive subpopulation, to complete our liquid biopsy approach. For this aim, we compared CTC enrichment technologies, such as an epitope-dependent, EpCAM-based system (CellSearch®; Menarini, Silicon Biosystems, Bologna, Italy), with an epitope-independent, microfluidic system (Parsortix; Angle Inc., Guildford, UK). Additionally, PD-L1 expression was analysed in the enriched CTCs using both technologies. Overall, cfDNA and CTC monitoring provides clinically relevant information to select patients who will benefit most from immunotherapy. To our knowledge, this study is the first to examine the association of combined levels of both circulating biomarkers with survival and the response to first-line pembrolizumab therapy in patients with metastatic NSCLC.

2. Materials and methods

2.1. Cell lines and culture

The lung cancer cell lines A549, NCI-H322 and NCI-H460 were purchased from the American Type Culture

Collection (ATCC® CCL-185™; Manassas, VA, USA) and routinely cultured in the ATCC-recommended growth medium at 37 °C, 5% CO₂ and 95% humidity. Cancer cell lines were treated with 100 ng·mL⁻¹ IFN- γ (Merck KGaA, Darmstadt, Germany) for 48 h to obtain different levels of PD-L1 expression.

2.2. Patients and blood sample collection

We designed a prospective study including patients with advanced NSCLC treated with pembrolizumab as first-line therapy between June 2017 and January 2021 at the Department of Medical Oncology of Complejo Hospitalario Universitario de Santiago de Compostela (Fig. 1). Fifty consecutive patients were recruited. Samples were collected from each patient at different time points: prior to the start of treatment (baseline) and 6 and 12 weeks after the first pembrolizumab dose (Fig. 1). One hundred and nine peripheral blood samples were obtained from patients. All individuals provided written informed consent prior to enrolling in the study, and the procedure was approved by Santiago de Compostela and Lugo Ethics Committee (Ref: 2017/538). The approved protocol was conducted according to the Declaration of Helsinki.

The efficacy of the treatment was evaluated based on RECIST1.1 criteria as follows: complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD). Progression-free survival (PFS) was defined as the time from the date of initial treatment until the date of disease progression, death or the last follow-up if progression or death had not occurred. Overall survival (OS) was defined as the time from the date of initial treatments until death or the last follow-up.

2.3. CfDNA isolation from plasma samples

Twenty millilitres of peripheral whole blood from patients with cancer was obtained by direct venipuncture and collected using CellSave tubes (Menarini, Silicon Biosystems, Bologna, Italy). Plasma was separated within 96 h after blood collection through two sequential centrifugation steps (10 min at 1600 *g* and 10 min at 6000 *g*; both at room temperature) and then stored at -80 °C until further processing. CfDNA was extracted from 3 mL of plasma using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) and a vacuum pump, according to the manufacturer's instructions.

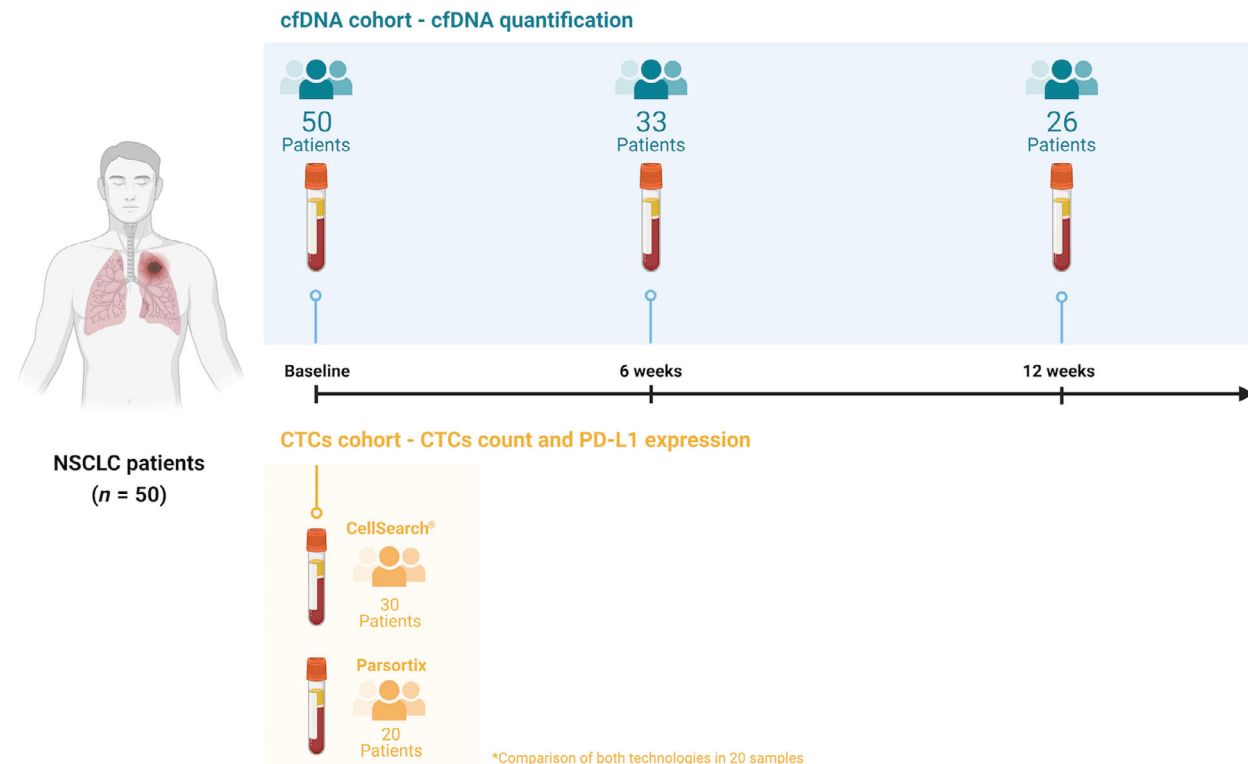


Fig. 1. Study schema and monitoring of the patient cohort, including patient enrolment and sample collection.

2.4. CfDNA quantification

Circulating free DNA yields were determined using the quantitative PCR (qPCR) method by analysing the telomerase reverse transcriptase (*hTERT*) single-copy gene (Thermo Fisher Scientific, Waltham, MA, USA), as previously reported [18]. The hydrolysis probe is located on chr. 5:1253373 with an 88-bp amplicon that maps within exon 16 of the *TERT* gene. qPCR was carried out in a final volume of 20 μ L consisting of 10 μ L of TaqMan Universal Mastermix (Thermo Fisher Scientific), 1 μ L of *hTERT* hydrolysis probe and 2 μ L of sample. Amplification was performed under the following cycling conditions using a QuantStudio™ 3 real-time PCR system (Thermo Fisher Scientific): 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C 15 s; and 60 °C for 1 min. Data were analysed with QUANTSTUDIO™ Design & Analysis software, version 2.5.1 (Thermo Fisher Scientific).

Each plate included a calibration curve and negative controls. The calibration curve was calculated based on a dilution series of standard human genomic DNA (Roche Diagnostics, Mannheim, Germany) fragmented into 184-bp fragments using a Covaris® E220-focused ultrasonicator (Covaris Inc., Woburn, MA, USA). gDNA was fragmented in a 6 × 16 mm microTUBE AFA Fibre Pre-Slit Snap-Cap (Covaris) using the following settings: 430-s duration, peak incident power of 175 Watts, duty factor of 10% and 200 cycles per burst. Fragment sizes were then determined using a TapeStation 4700 (Agilent, Santa Clara, CA, USA) and High Sensitivity DNA ScreenTape® (Agilent). Each sample was analysed in duplicate, and the final concentration was calculated by interpolation of the mean of the quantification cycle (Cq) with the calibration curve. Values with a Cq confidence interval less than 0.95 were discarded. Moreover, only assays with R^2 values greater than 0.98 for the standard curve and with an efficiency $\geq 88.8\%$ were used.

2.5. Spiked experiments

The assays to evaluate PD-L1 expression on CTCs were tested using the cancer cell lines A549, NCI-H322 and NCI-H460 spiked in whole blood from the healthy volunteers recruited for this study. The protocol employed was described previously [12]. Briefly, cells were trypsinized to approximately 80% confluence, and then, 200 cells were added manually (with a calculated pipetting error of 10%) to a total of 7.5 mL of blood from healthy donors collected in CellSave tubes (Menarini, Silicon Biosystems). The samples were analysed using the CellSearch® and Parsortix systems, and

two tubes of the same sample were analysed with both technologies. All spiked samples were enriched within 48 h of collection.

2.6. Analysis of PD-L1 expression on CTCs isolated using CellSearch®

A total of 7.5 mL of peripheral whole blood samples was collected in CellSave tubes (Menarini, Silicon Biosystems) for CTC enumeration using the CellSearch® system (Menarini, Silicon Biosystems). A CellSearch® CXC Kit (Menarini, Silicon Biosystems) was used for these specific experiments, including ferrofluids coated with epithelial cell-specific anti-EpCAM antibodies to immunomagnetically enrich epithelial cells; a mixture of antibodies against cytokeratins (CKs) 8, 18 and 19 conjugated to fluorescein (FLU); an anti-CD45 mAb conjugated to allophycocyanin (APC); and nuclear dye 4',6-diamidino-2-phenylindole (DAPI) to fluorescently label the cells. The open 4th antibody position of the CellSearch® system was used to evaluate PD-L1 expression according to the 'Guideline for the Use and Optimization of User Defined Markers: CellSearch® Epithelial Cell Kit and CellSearch® CXC Kit, version 1.0' for its optimization. We employed the anti-human B7-H1/PD-L1 phycoerythrin (PE)-conjugated antibody (Cat N° FAB1561P; R&D Systems, Minneapolis, MN, USA) at a final concentration of 20 μ g·mL⁻¹, as described previously [19]. CTCs were identified as EpCAM⁺, CK⁺, CD45⁻ and DAPI⁺, and PD-L1 expression was recorded for each CTC (presence or absence) by comparison with the PD-L1 expression levels in the cell lines. The specificity of the staining was confirmed by the lack of signals detected with our negative cell line, A549. As positive control, we employed the cell line NCI-H460 stimulated with IFN- γ .

2.7. Analysis of PD-L1 expression on CTCs isolated using the Parsortix system

A total of 7.5 mL of peripheral whole blood was collected in CellSave tubes (Menarini, Silicon Biosystems) and loaded into a Parsortix microfluidic device (Angle Inc., Guildford, UK), as described previously [12]. Briefly, CTCs were then enriched from blood samples in disposable Parsortix cassettes with a size of 6.5 μ m (GEN3D6.5; Angle Inc., Guildford, UK) and at 99 mbar of pressure, according to the manufacturer's guidelines. CTCs were trapped in the Parsortix cassette due to their large size and lower compressibility than the remaining blood cells. After separation, we fixed the sample with 4% paraformaldehyde and carried out

on-cassette staining with selected antibodies according to the manufacturer's guidelines, followed by fluorescence microscopy detection (Leica DMI8; Leica Microsystems, Wetzlar, Germany). The selected antibodies included Alexa Fluor 647-conjugated CD45 (35-Z6, sc-1178; Santa Cruz, CA, USA) at a final concentration of $4 \mu\text{g}\cdot\text{mL}^{-1}$ to detect white blood cells; the same anti-human B7-H1/PD-L1 PE-conjugated antibody (R&D Systems) used in CellSearch[®] systems at a final concentration of $20 \mu\text{g}\cdot\text{mL}^{-1}$ to detect PD-L1 expression; Alexa Fluor 488-conjugated pan-CK (A4-108-C100; EXBIO Praha, Vestec, Czech Republic) at a concentration of $1.33 \mu\text{g}\cdot\text{mL}^{-1}$; and DAPI to fluorescently label the cells. CTCs were identified as CK+, CD45- and DAPI+, and PD-L1 expression on CTCs was determined (presence or absence) according to the results obtained from cell line-spiked samples. We employed Leica Application Suite X (LAS X) (Leica Microsystems) to identify the fluorescence intensity in each single cell, and PD-L1 expression on CTCs was determined (presence or absence) according to the results obtained from cell line-spiked samples. The specificity of the staining was confirmed by the lack of signals detected with our negative cell line, A549. As positive control, we employed the cell line NCI-H460 stimulated with IFN- γ .

2.8. PD-L1 immunohistochemistry and scoring

PD-L1 IHC was carried out on 4- μm sections of FFPE tumour tissue samples using Dako PD-L1 IHC 28-8 PharmaDx (Agilent). The test was performed using the EnVision FLEX visualization system on the Dako Autostainer Link 48 and Dako PT Link Pretreatment Module (Agilent). A minimum of 100 viable tumour cells must be present for evaluation. PD-L1 expression was evaluated only in tumour cells. Scoring was determined according to the tumour proportion score (TPS), which is defined as the percentage of positive viable tumour cells among all viable tumour cells evaluated. A tumour cell was defined as positive for PD-L1 staining whenever any partial or complete membranous staining was detected. The percentage of PD-L1-positive tumour cells was assessed as previously described [20]. Slides were assessed independently by two pathologists.

2.9. Statistical analysis

Statistical analyses were performed using R version 4.0.2. The Spearman correlation coefficients were calculated to assess the correlation between the PD-L1 TPS and PD-L1 status of CTCs. The kappa test was used to determine the concordance with a 95% confidence

interval (CI). We dichotomized the CTC PD-L1 counts as positive and negative and categorized PD-L1 expression (PD-L1 tissue expression, 80–100% vs < 80%) to calculate the kappa coefficients. Receiver operating characteristic (ROC) curves were constructed, and the area under the ROC curve (AUC) with 95% CIs was obtained to evaluate the thresholds of baseline *hTERT* cfDNA levels for OS and PFS analyses. The AUC and the 95% CIs for the sensitivity and specificity were estimated using the pROC package in R software [21]. Univariate and multivariate Cox regression analyses were performed using the survival package in R [22], and a Kaplan–Meier analysis was then performed. The associations between CTCs and *hTERT* cfDNA with the best response were estimated using Fisher's exact test. We also used Fisher's exact test to compare the association between CTC counts and the response to therapy.

3. Results

3.1. Study population

The characteristics of the patients enrolled in the study are summarized in Table 1. The median age was 63.3 years (range: 45–79), and the majority of patients were males (74%), and current or former smokers (86%) and had tumours with an adenocarcinoma histology (72%). Eighty per cent of patients had an Eastern Cooperative Oncology Group Performance Status (ECOG PS) of 1–2, and 30% of patients had more than two metastases. Thirty-four per cent of patients exhibited PD-L1 expression in the tissue at a level $\geq 80\%$, and the median number of pembrolizumab treatment cycles was 6 (range 1–35 cycles). The median PFS and OS were 10.47 and 19.13 months, respectively, in the 50 patients with NSCLC. The objective response rate (ORR: complete response or partial response during ≥ 6 cycles) was 46.0%, with 1 complete and 22 partial responses.

3.2. Circulating free DNA analyses

3.2.1. Prognostic and predictive value of cfDNA levels at baseline

We next evaluated the role of cfDNA levels as a prognostic biomarker for pembrolizumab treatment outcomes in our cohort of patients with NSCLC. We employed the *hTERT* qPCR assay to determine the cfDNA levels. The cohort was dichotomized into two groups (high and low levels) according to a threshold calculated based on the baseline *hTERT* cfDNA levels observed in our cohort

Table 1. Demographics and clinical characteristics of the patients at baseline. SD, standard deviation; NSCLC-NOS, non-small-cell lung cancer-not otherwise specified.

Baseline characteristics	Patients <i>n</i> ^a (%)
Mean age (years) ± SD, range	63.3 ± 8.3, 45–79
Sex	
Female	13 (26)
Male	37 (74)
Smoking	
Smoker	31 (62)
Former smoker	12 (24)
Never	7 (14)
Histology	
Adenocarcinoma	44 (88)
Squamous cell carcinoma	4 (8)
NSCLC-NOS	2 (4)
ECOG PS	
0	10 (20)
1	34 (68)
2	6 (12)
PD-L1 expression in the tissue	
< 80%	33 (66)
≥ 80%	17 (34)
Number of metastatic sites	
≤ 2	35 (70)
> 2	15 (30)
Pembrolizumab treatment	
Monotherapy	37 (74)
In combination with chemotherapy	13 (26)

^a*n* = 50 at baseline.

(Table S1). These levels were log10-transformed by choosing 7.665 (2132.39 in genome equivalents·mL⁻¹, GE·mL⁻¹ plasma) and 7.638 (2075.59 in genome equivalents·mL⁻¹, GE·mL⁻¹ plasma) for PFS and OS analyses, respectively, after considering sensitivity and specificity based on ROC curve analyses (Table S1). Patients with high baseline *hTERT* cfDNA levels had a significantly shorter PFS (*P*-value < 0.01; hazard ratio, 2.89; 95% CI, 1.30–6.45) and OS (*P*-value = 0.005; hazard ratio, 3.26; 95% CI, 1.43–7.47) than those with low baseline levels (Fig. 2A,B and Table 2).

The median OS was 28.4 months in the low baseline *hTERT* cfDNA group and 4.9 months in the high baseline *hTERT* cfDNA group, whereas the median PFS was 14.6 and 5.1 months in the two cfDNA categories (low vs. high baseline levels, respectively) (Fig. 2A,B). Considering various clinical and demographic variables (ECOG PS, sex, age, PD-L1 expression in the tissue, number of metastases and smoking status), univariate and multivariate Cox regression analyses of PFS and OS were performed (Table 2). In this analysis, *hTERT* cfDNA levels did not show value as an independent

predictive biomarker of PFS and OS, with the number of metastases representing the main independent factor explaining the PFS rates.

3.2.2. Monitoring *hTERT* cfDNA levels and the response to therapy

We next investigated the value of *hTERT* cfDNA kinetics as a prognostic biomarker during pembrolizumab treatment (Figs 3 and 4). Blood samples were collected longitudinally, before the initiation of therapy, and at 6 and 12 weeks after the onset of pembrolizumab therapy (Fig. 1). No significant differences were observed in the global cfDNA levels between any group (Fig. S1A). We monitored and investigated the relationship between *hTERT* cfDNA levels and the response to therapy in our patient cohort (Fig. 3). We did not find any association between the cfDNA levels at baseline and the response to pembrolizumab therapy (Fig. S1B). However, after considering the changes from baseline to 12 weeks, we found an association with treatment response (Fig. 4A). We observed two patterns: an increase in *hTERT* cfDNA levels at 12 weeks (*n* = 14) and a decrease in *hTERT* cfDNA levels at 12 weeks (*n* = 12), with a median PFS of 6.8 and 4.6 months, respectively (Fig. 4B).

We also analysed the risk of disease progression at each time point. According to the ROC curve analysis, the thresholds of cfDNA levels at baseline, 6 weeks and at 12 weeks were chosen for the PFS analysis (Table S1). High levels of *hTERT* cfDNA at 12 weeks were a strong predictor of the risk of disease progression (*P*-value < 0.005, odds ratio = 18, 95% CI 2.5–131.3) (Fig. 4C). Fifteen patients showed high levels at 12 weeks, and 12 of them (80%) developed progressive disease compared with 2 of the 11 patients (18.2%) with low levels. Moreover, at each time point, patients were divided into favourable and unfavourable risk groups after considering their changes in *hTERT* cfDNA levels. The median PFS was 7.07 months for the unfavourable risk group based on the changes between baseline and 12 weeks, whereas median PFS was not reached for the favourable risk group (*P*-value < 0.01; hazard ratio, 6.8; 95% CI, 1.5–30.5) (Fig. 4D).

3.3. Circulating tumour cell analyses

3.3.1. EpCAM-dependent versus antigen-independent CTC isolation to quantify CTCs and characterize the PD-L1 status

In addition to monitoring *hTERT* cfDNA levels, we analysed CTC levels, as they represent a more

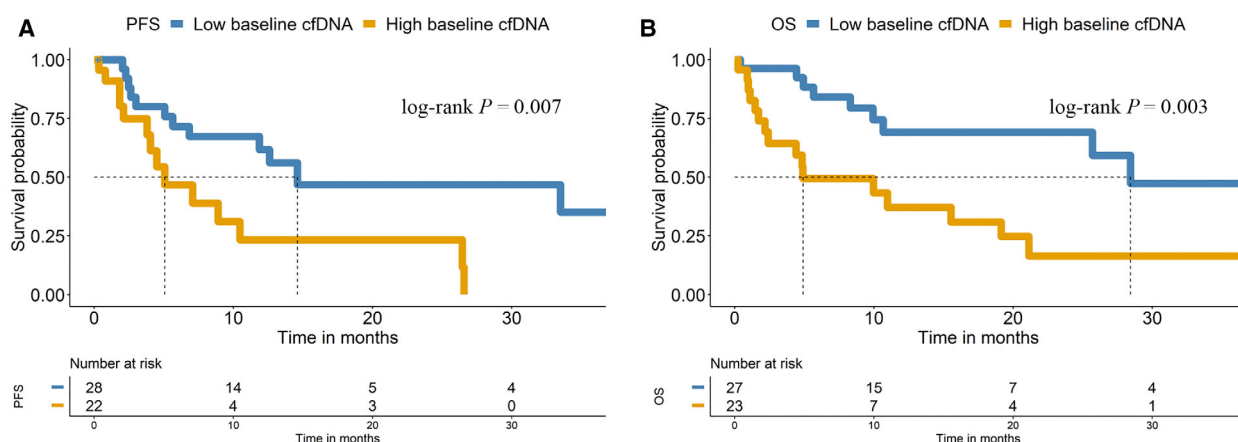


Fig. 2. Kaplan–Meier survival analysis of *hTERT* cfDNA levels at baseline. Kaplan–Meier plots of PFS (A) and OS (B).

biological feature of the tumour. Therefore, we evaluated two different technologies, the EpCAM-based CellSearch® system and the label-independent microfluidic Parsortix system, to quantify CTCs and assess the expression of PD-L1 and to determine the advantages of the non-EpCAM-dependent isolation method. First, we categorized the presence or absence of PD-L1 on single CTCs to grade its expression in each cell line (with known gradual increases in PD-L1 protein expression) using both approaches (Fig. 5A,B). We used preserved blood samples from healthy controls spiked with three lung cancer cell lines (A549, *no expression*; NCI-H322, *low-medium expression*; and NCI-H460, *medium-high expression*) representative of the variability of PD-L1 expression (Fig. S2).

Next, peripheral blood samples were collected from patients prior to treatment to analyse the presence of CTCs and their PD-L1 expression (Fig. 5C,D) as potentially valuable prognostic and predictive biomarkers. In 20 samples from patients, we compared CTC enumeration using both strategies (CellSearch® and Parsortix systems) and evaluated the performance and concordance between them (Fig. S3A,B and Table S2). Using the CellSearch® system, we detected ≥ 1 CTC in 50% (10/20) of samples (range 1–168; mean = 9.8), while using the Parsortix system, 35% (7/20) of samples had ≥ 1 CTC (range 1–56; mean = 4.7). Regarding the capacity to detect PD-L1 expression in CTCs, we detected PD-L1-positive CTCs in 2 of 17 samples (in 2/10 samples with CTCs) using the CellSearch® system. Compared with the label-independent system, we observed PD-L1-positive CTCs in 7 of 20 samples (in 7/7 samples with CTCs) using the Parsortix system. When we compared the concordance of both technologies, we found that kappa scores for the number of CTCs and PD-L1-positive CTCs presented

negative values for both technologies (−0.1 and 0.14, respectively), showing no correlation between them.

3.3.2. Correlation of the PD-L1 status in CTCs and tissue samples

We also compared the PD-L1 status of CTCs using CellSearch® and Parsortix systems with PD-L1 expression in the primary tumour biopsy obtained at the initial diagnosis (Fig. S4). The PD-L1 TPS in biopsies did not correlate with the percentage of PD-L1-positive CTCs at the initial diagnosis (P -value = 0.59 and P -value = 0.71 for the CellSearch® and Parsortix systems, respectively). The mean time between tissue biopsy and liquid biopsy sample collection was 38.6 days (range 9–78). Kappa scores for the PD-L1 status in CTCs and PD-L1 expression in tissue were low for both technologies. Thus, using the CellSearch® system, the concordance rate was 26.7% with Cohen's kappa of 0.04, while with the Parsortix system, the concordance rate was higher, 47.1% with Cohen's kappa of 0.14.

3.3.3. Prognostic and predictive values of CTC enumeration at baseline

We evaluated the role of CTC enumeration in prognosticating disease progression in response to pembrolizumab treatment in our cohort of patients with advanced NSCLC. For this purpose, we performed CTC enumeration at baseline in 30 patients using the CellSearch® system and 20 patients using Parsortix. Patients with CTCs identified using the CellSearch® system had significantly shorter PFS and OS than patients who had no CTCs (P -value < 0.05) (Fig. 6A,B). The median PFS was 12.6 and 3 months in the two groups

Table 2. Univariate and multivariate Cox regression analyses of cfDNA levels, CTC counts and clinical parameters. ECOG, Eastern Cooperative Oncology Group Performance Score. The levels of cfDNA were determined as low (< cut-off) or high ($\geq$ cut-off) based on the cut-off obtained from the ROC curve analyses.

Variable	Univariate		Multivariate	
	P-value	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)
PFS				
Baseline log cfDNA (high vs. low cfDNA, $n = 50$)	0.009	2.89 (1.30–6.45)	0.80	1.18 (0.28–5.01)
Baseline CTC count, CellSearch (≥ 1 vs. 0, $n = 30$)	0.04	2.97 (1.04–8.45)	0.006	9.36 (1.88–46.6)
ECOG (≥ 1 vs. 0, $n = 50$)	0.20	2.09 (0.72–6.08)	0.70	1.40 (0.24–8.15)
PD-L1 expression in the tissue (≥ 80 vs. < 80 , $n = 50$)	0.40	0.68 (0.27–1.76)	0.13	0.37 (0.10–1.36)
Sex (male vs. female, $n = 50$)	0.40	0.71 (0.32–1.57)	0.04	0.23 (0.05–0.97)
Age (years, $n = 50$)	0.60	0.99 (0.93–1.04)	0.30	1.05 (0.96–1.16)
Number of metastasis (> 2 vs. ≤ 2 , $n = 50$)	0.04	2.39 (1.03–5.55)	0.006	9.21 (1.87–45.3)
Smoking (yes vs. no, $n = 50$)	0.40	0.68 (0.25–1.82)	0.03	11.4 (1.25–104)
Combined changes in CTC and cfDNA levels ^a				
Group 1 (CTCs < 1 and a low cfDNA level, $n = 12$)	–	Reference	–	–
Group 2 (CTCs < 1 and a high cfDNA level or CTCs ≥ 1 and a low cfDNA level, $n = 10$)	0.005	5.37 (1.66–17.4)	0.009	13.1 (1.91–90.1)
Group 3 (CTCs ≥ 1 and a high cfDNA level, $n = 8$)	0.05	4.12 (0.98–17.4)	0.01	14.5 (1.76–119)
Combined changes in CTC and cfDNA levels ^a				
Group A (CTCs < 1 and a low cfDNA level, $n = 12$)	–	Reference	–	–
Group B (CTCs < 1 and a high cfDNA level or CTCs ≥ 1 and a low cfDNA level or CTCs ≥ 1 and a high cfDNA level, $n = 18$)	0.006	4.99 (1.60–15.6)	0.005	13.6 (2.17–85.3)
OS				
Baseline log cfDNA (high vs. low cfDNA levels, $n = 50$)	0.005	3.26 (1.43–7.47)	0.90	1.13 (0.29–4.46)
Baseline CTC count, CellSearch (≥ 1 vs. 0, $n = 30$)	0.03	2.71 (1.11–6.64)	0.01	5.41 (1.42–20.6)
ECOG (≥ 1 vs. 0, $n = 42$)	0.06	4.05 (0.95–17.2)	0.70	1.51 (0.25–8.99)
PD-L1 expression in the tissue (≥ 80 vs. < 80 , $n = 50$)	0.50	1.36 (0.60–3.10)	0.50	0.64 (0.20–2.04)
Sex (male vs. female, $n = 42$)	0.70	1.18 (0.49–2.86)	0.20	0.42 (0.10–1.76)
Age (years, $n = 42$)	0.80	1.01 (0.95–1.07)	0.20	1.07 (0.98–1.17)
Number of metastasis (> 2 vs. ≤ 2 , $n = 42$)	0.005	3.12 (1.41–6.87)	0.001	9.08 (2.35–35.1)
Smoking (yes vs. no, $n = 42$)	0.60	0.77 (0.29–2.05)	0.02	20.5 (1.54–273)
Combined changes in CTC and cfDNA levels ^a				
Group 1 (CTCs < 1 and a low cfDNA level, $n = 12$)	–	Reference	–	–
Group 2 (CTCs < 1 and a high cfDNA level or CTCs ≥ 1 and a low cfDNA, $n = 10$)	0.14	2.33 (0.76–7.15)	0.50	1.90 (0.33–11.0)
Group 3 (CTCs ≥ 1 and a high cfDNA level, $n = 8$)	0.02	4.24 (1.29–14.0)	0.01	6.39 (1.37–29.8)
Combined changes in CTC and cfDNA levels ^a				
Group A (CTCs < 1 and a low cfDNA level, $n = 12$)	–	Reference	–	–
Group B (CTCs < 1 and a high cfDNA level or CTCs ≥ 1 and a low cfDNA level or CTCs ≥ 1 and a high cfDNA level, $n = 18$)	0.04	2.91 (1.05–8.07)	0.05	4.02 (0.97–16.6)

^aMultivariate Cox regression model including sex, age, Eastern Cooperative Oncology Group Performance Score, PD-L1 expression in the tissue, number of metastases and smoking status.

(0 vs. ≥ 1 CTC, respectively). The median OS of the CTC-positive group was 4.9 months, whereas the median OS was 21.13 months for the CTC-negative group. In the multivariate regression analysis (Table 2), we confirmed that CTC positivity using the CellSearch[®] system was an independent predictive biomarker of PFS and OS (hazard ratio, 5.75; 95% CI, 1.35–24.5, P -value < 0.05 and hazard ratio, 4.59; 95% CI, 1.32–16.0, P -value < 0.05 , respectively). We observed no significant results for PFS or OS when analysing CTC counts with

the Parsortix system and evaluating PD-L1-positive CTCs, regardless of the technology employed (CellSearch[®] and Parsortix systems).

We also investigated the relationship between the CTC counts detected using both approaches and the response to pembrolizumab therapy in our patient cohort. Significant differences were observed between the CTC count detected with the CellSearch[®] system and the achievement of a complete or partial response and disease progression (P -value < 0.05) (Fig. 6C).

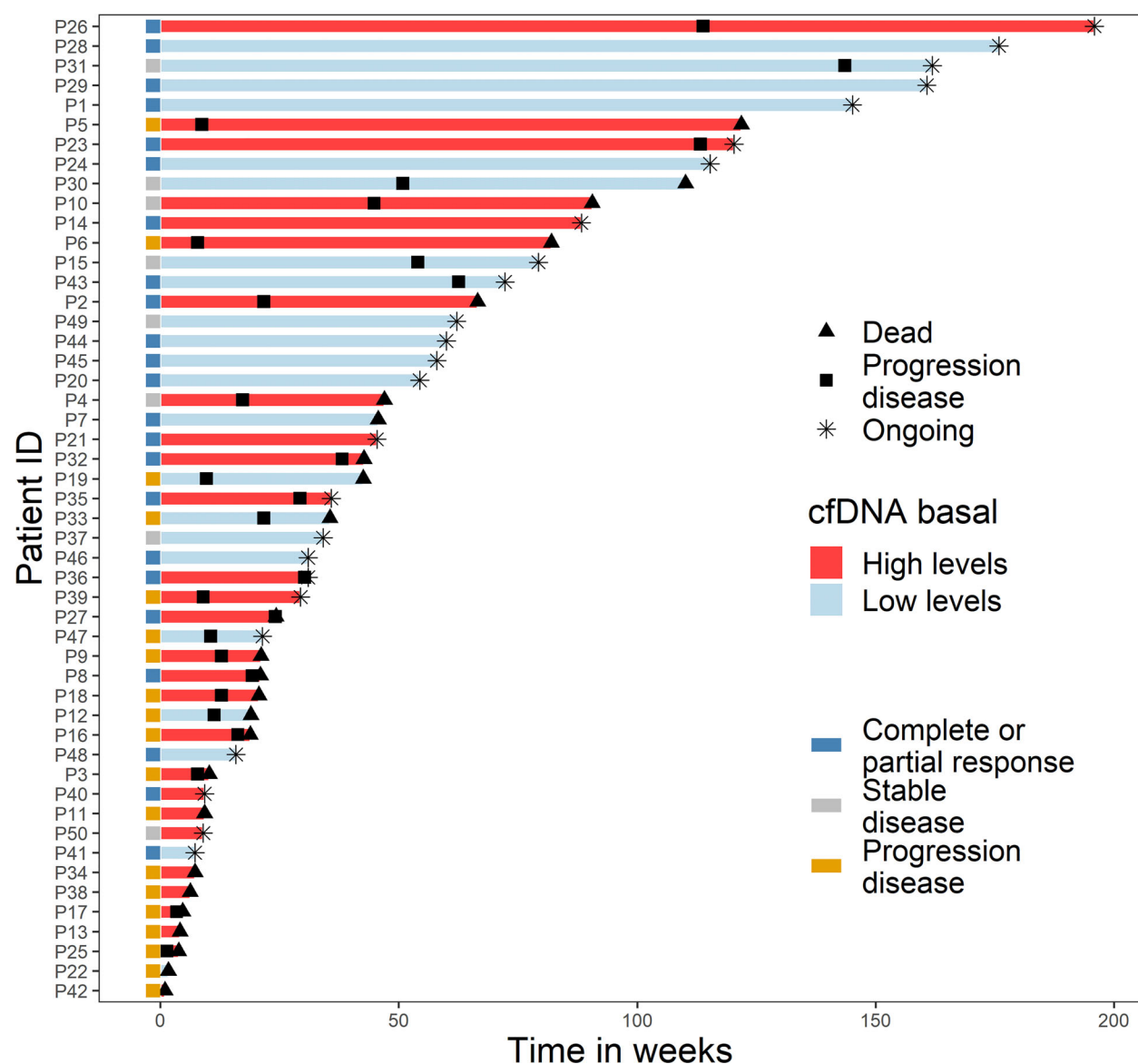


Fig. 3. Clinical course for patients during pembrolizumab treatment. Swimmer plots for each patient ($n = 50$) showing the levels of *hTERT* cfDNA at baseline (red colour indicates high *hTERT* cfDNA levels, and blue colour indicates low *hTERT* cfDNA levels). The total length of each bar indicates the duration of survival from start of pembrolizumab treatment. Left, squares are coloured according to the response based on RECIST1.1 criteria.

The ORR was similar between patients with undetectable CTCs compared with patients with detectable PD-L1-positive or PD-L1-negative CTCs using CellSearch[®] and Parsortix technologies (Table S3).

3.4. Clinical potential of combined CTC and cfDNA analyses

We also evaluated the joint effect of baseline CTC counts using the CellSearch[®] system and *hTERT* cfDNA levels.

First, we considered three risk subgroups: (a) CTCs < 1 and low cfDNA baseline levels; (b) CTCs < 1 and high cfDNA baseline levels or CTCs ≥ 1 and low cfDNA baseline levels; and (c) CTCs ≥ 1 and high cfDNA baseline levels. Multivariate Cox analyses confirmed that the combined analyses of CTCs using the CellSearch[®] system and *hTERT* cfDNA levels were independent predictive biomarkers of PFS (P -value < 0.01; hazard ratio, 11.6; 95% CI, 2.04–66.1; P -value < 0.05; hazard ratio, 14.3; 95% CI, 1.7–117) (Table 2).

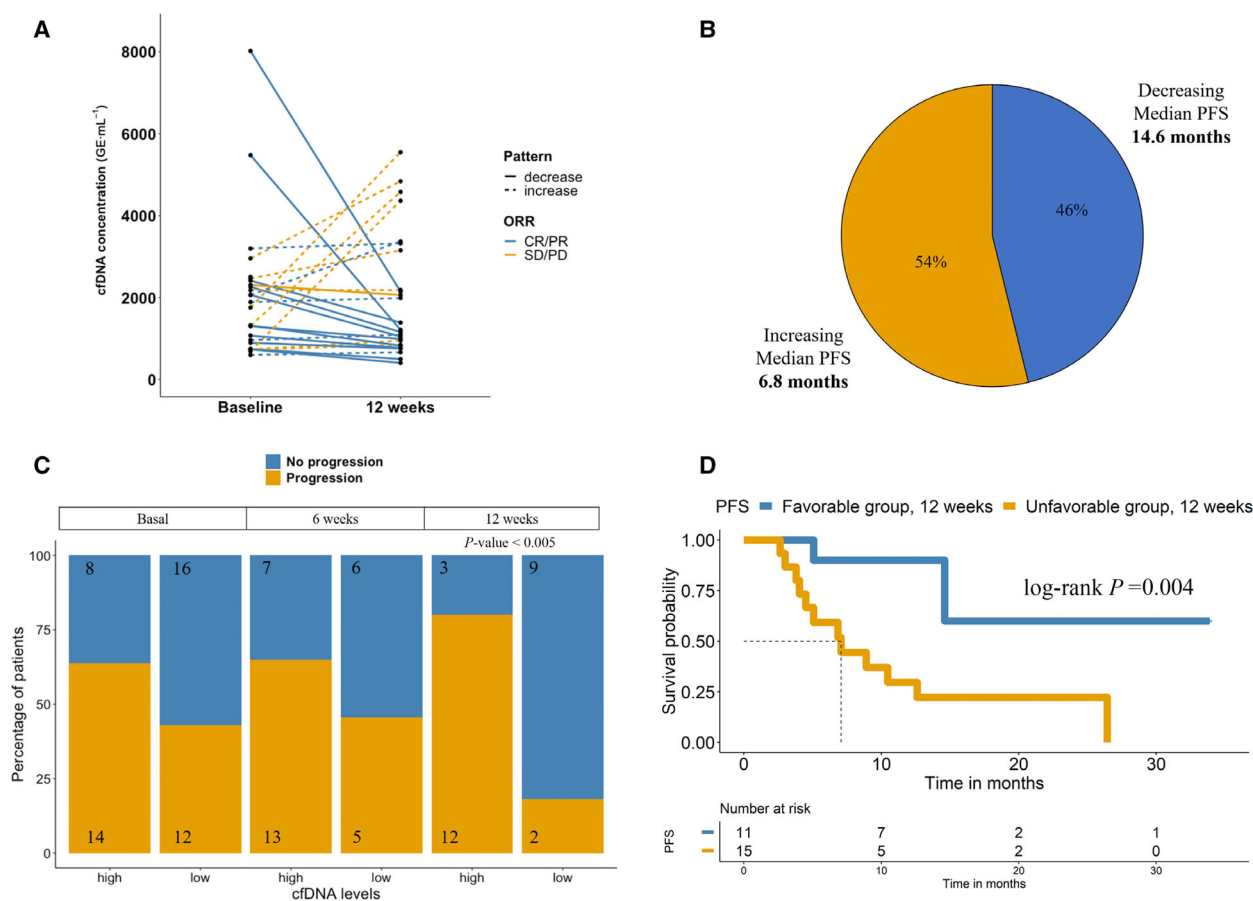


Fig. 4. *hTERT* cfDNA changes from baseline to 12 weeks. (A) *hTERT* cfDNA concentrations for the two cfDNA patterns (increase/decrease at 12 weeks) and showing the response to therapy. (B) Percentage of patients and median PFS for each cfDNA pattern; (C) Proportion of patients with high and low levels baseline, 6 and 12 weeks. P -value was calculated by Fisher's exact test. (D) Kaplan–Meier plot of PFS of the favourable/unfavourable changes at 12 weeks. Groups are composed of patients with unfavourable changes: both high *hTERT* cfDNA levels at baseline (cut-off ≥ 7.665) and at 12 weeks (cut-off ≥ 7.026), or low levels at baseline (cut-off < 7.665) and high levels at 12 weeks (cut-off ≥ 7.026). The low-risk group (patients with favourable changes) is composed of patients with low *hTERT* cfDNA levels at baseline (cut-off < 7.665) and low levels at 12 weeks (cut-off < 7.026) or high levels at baseline (cut-off ≥ 7.665) and low levels at 12 weeks (cut-off < 7.026). CR/PR, complete response/partial response; SD/PD, stable disease/progression disease.

Subsequently, we considered only two risk subgroups to simplify the analysis: (A) CTCs < 1 and low cfDNA baseline levels; and (B) CTCs < 1 and high cfDNA baseline levels or CTCs ≥ 1 and low cfDNA baseline levels or CTCs ≥ 1 and high cfDNA baseline levels. Patients from subgroup A (undetectable CTCs using CellSearch[®] and low cfDNA levels) had significantly shorter PFS (P -value < 0.01 ; hazard ratio, 4.99; 95% CI, 1.6–15.6) and OS (P -value < 0.05 ; hazard ratio, 2.9; 95% CI, 1.1–8.1) than patients from subgroup B (Table 2). The median PFS was not reached in group A (low cfDNA levels and undetectable CTCs at baseline), whereas the median PFS was 4.0 months in group B. Similarly, the median OS was not reached in group A, whereas the median OS was 4.9 months in

group B (Fig. 7A,B). Overall, the hazard ratios and P -values that emerged from the univariate and multivariate Cox analyses suggest a combinatory effect of both markers as early predictors of disease progression (Table 2).

Additionally, patients with CTCs identified using the CellSearch[®] system and high cfDNA levels at baseline showed a trend towards a poorer response to pembrolizumab therapy (Fig. S5).

4. Discussion

Liquid biopsy represents a promising tool for the diagnosis, selection and monitoring of response to ICI treatment in the context of NSCLC. Currently, PD-L1

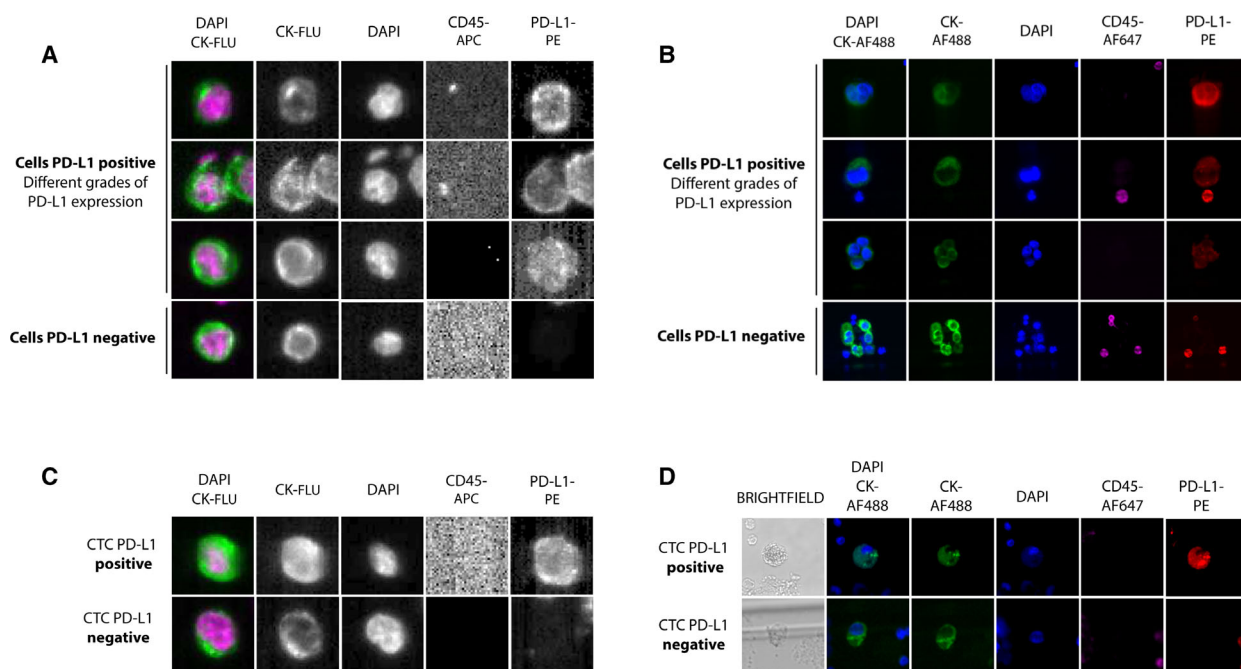


Fig. 5. (A, B) Detection of PD-L1 expression after spiking cancer cell lines in healthy blood and analysis of them with the CellSearch® and Parsortix systems, respectively, and representative images of different grades of PD-L1 expression. (C) Representative images of CTCs detected with the CellSearch® system in patients with NSCLC. Samples were subjected to immunostaining with DAPI, CD45 (APC), cytokeratins (FLU) and PD-L1 (PE). (D) Representative images of CTCs detected with the Parsortix system in patients with NSCLC. Samples were subjected to immunostaining with DAPI, CD45 (AF647), cytokeratins (AF488) and PD-L1 (PE). NCI-H460 stimulated with IFN- γ shown high expression, NCI-H460, medium expression, NCI-H322, low-medium expression, and A549, no expression.

is the best studied biomarker for ICI treatment selection, exhibiting a higher probability of response with higher expression of PD-L1 [13]. In patients with advanced NSCLC and PD-L1 expression on at least 50% of tumour cells, pembrolizumab results in significantly longer PFS and OS [4], and thus, the determination of PD-L1 expression in tissue samples is the reference factor for the selection of ICI treatments. However, in clinical practice, patients with high levels of PD-L1 may not respond to ICI treatment; in contrast, in the absence of PD-L1, a clinical benefit may be obtained from the use of PD-1 or PD-L1 checkpoint inhibitors [13]. A high tumour mutational burden (TMB), which is associated with high levels of neoantigens, represents another potential candidate biomarker that would drive the choice of treatment [4], although this biomarker has not been translated into the clinic due to conflicting results among studies [23–28]. Notably, these two markers have been mainly examined in tissue samples.

In the present study, we analysed the value of *hTERT* cfDNA and CTC analyses as potential prognostic and predictive noninvasive biomarkers to discriminate patients who will benefit from ICIs. We

explored the clinical value of monitoring *hTERT* cfDNA levels and detected the presence of CTCs, including PD-L1 characterization, in a homogeneous cohort of patients with metastatic NSCLC receiving first-line treatment with pembrolizumab as a monotherapy or in combination with chemotherapy.

Circulating free DNA has already been proposed as a prognostic and predictive biomarker in NSCLC [29–31], but few studies have reported the cfDNA concentration as a predictive marker of the immunotherapy response. Although cfDNA represents promising material, standardized protocols for the processing and total quantification are still missing [32]. In our work, we propose the qPCR method to quantify *hTERT* cfDNA because it is a sensitivity and cost-effective assay and based on previous results obtained in lung cancer patients [15,18,33]. Alama *et al.* [15] reported that patients with NSCLC who were treated with a 2nd or higher line of nivolumab and with cfDNA level below their cohort median values survived significantly longer than those with a cfDNA level above this threshold. In another study, patients with NSCLC treated mainly with second-line therapy with nivolumab and with low ctDNA concentrations at the first evaluation showed a

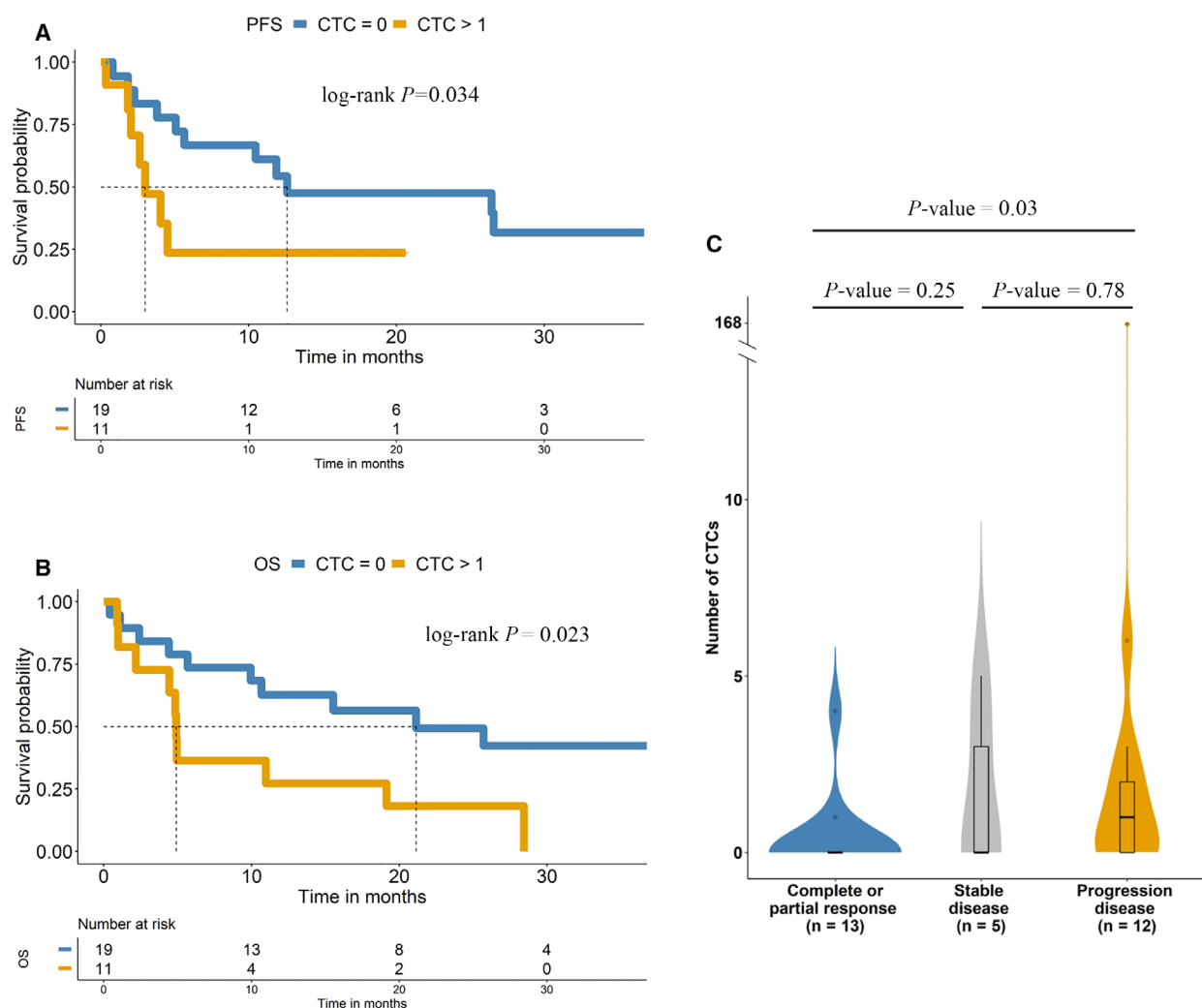


Fig. 6. Kaplan–Meier survival analysis of CTCs at baseline. Kaplan–Meier plots of PFS (A) and OS (B). (C) Comparison of the response to pembrolizumab based on CTC detection using CellSearch®. P -value was calculated by Fisher's exact test.

long-term benefit [16]. Notably, numerous studies have described the presence of somatic mutations in plasma cfDNA and their association with tumour response and survival [34–36]. Recently, an early decrease or total clearance of ctDNA levels after pembrolizumab administration identified subsets of patients with advanced solid tumours who had a good prognosis [37,38], regardless of the tumour type, PD-L1 status or TMB. In a previous study, changes in the ctDNA concentration were reported as a predictor of a durable response in patients treated with anti-PD-1 drugs [39], where the persistence of ctDNA exerted a detrimental effect. Goldberg *et al.* [40] also suggested that cfDNA levels may be an early marker of therapeutic efficacy, predicting prolonged survival in patients treated with ICIs for NSCLC. Based on these findings, serial ctDNA

analyses could serve as a generalizable monitoring strategy for patients treated with ICIs, but researchers have not clearly determined whether this approach is transferable to cfDNA, which is clearly easier to analyse.

Regarding the on-treatment cfDNA levels, data are scarce, although cfDNA levels appear to be decreased in response to effective treatments [41–43]. Our study prospectively evaluated the value of *hTERT* cfDNA kinetics as a prognostic biomarker during pembrolizumab treatment. Patients with NSCLC presenting high *hTERT* cfDNA levels at baseline or unfavourable changes from baseline to 12 weeks had a significantly greater risk of disease progression. Importantly, although the concentration of cfDNA can vary among individuals, depending on physiological factors and tumour characteristics [44,45], the cfDNA analysis

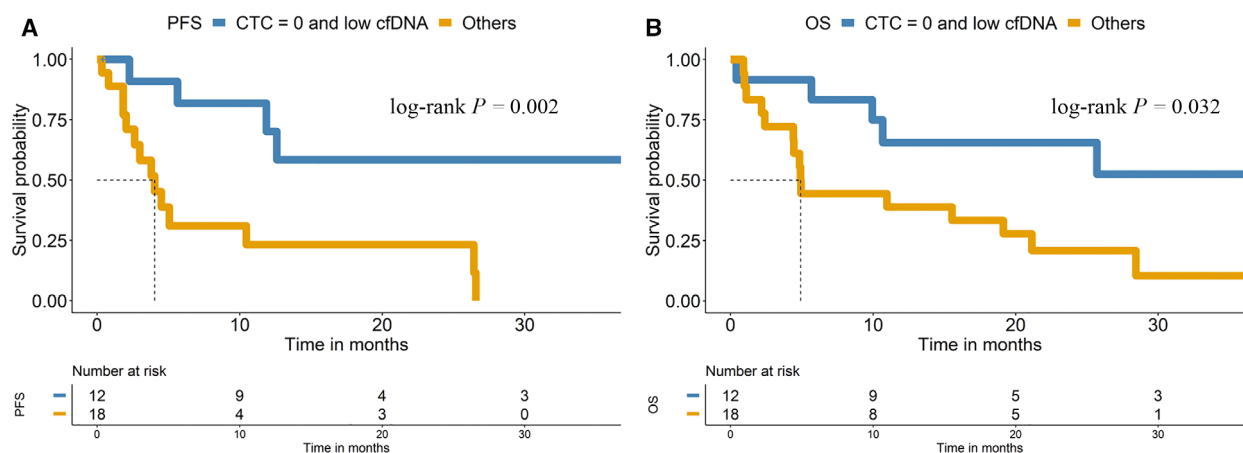


Fig. 7. CTCs and *hTERT* cfDNA correlate with the prognosis of patients with NSCLC treated with pembrolizumab. (A) Kaplan–Meier survival plot of PFS based on the combination of cfDNA and CTC levels at baseline. (B) Kaplan–Meier survival plot of OS based on the combination of cfDNA and CTC levels at baseline.

has clear advantages compared with the ctDNA determination, such as its feasibility, low detection cost and reproducibility. Therefore, our results reinforced the potential to monitor *hTERT* cfDNA dynamics in patients with NSCLC treated with ICIs, providing a simple tool to better anticipate the response to treatment.

We also investigated CTC levels using two CTC enrichment technologies, the EpCAM-based CellSearch® system (approved by the Food and Drug Administration (FDA) for the prognostic assessment of CTCs in patients with metastatic breast, colon and prostate cancer [46–48]) and the microfluidic epitope-independent-based method Parsortix system to complete our liquid biopsy approach. The CellSearch® system is the only FDA-approved device for CTC enumeration in some cancer types, although other immunomagnetic-based strategies have been developed for CTC enrichment [49,50]. The Parsortix system uses a combination of size-based and microfluidic-based enrichment approaches to separate CTCs from blood samples and solve the dependence on a single biomarker, EpCAM, in this case, allowing the detection of CTCs with a more mesenchymal phenotype. In our study, the detection rate using the CellSearch® system (50%) was higher than that using the Parsortix system (35%), and many differences at the individual level were observed when comparing the results of both strategies for the same patient, reinforcing the isolation of different CTC types with both technologies.

In a previous comparative study of patients with NSCLC, Janning *et al.* [51] reported a higher detection rate using the Parsortix than the CellSearch® system in patients with NSCLC receiving different therapy

regimens, attributing the difference to the heterogeneity and low EpCAM expression of some CTCs and therefore to the inability of EpCAM-based CellSearch® to detect certain subpopulations. Our cohort was a more homogeneous cohort, with patients naive to previous treatments. This last factor can favour the epithelial characteristics of CTCs, since the EMT is induced as a result of drug resistance in NSCLC [52–55]. However, we analysed only CK-positive CTCs in combination with PD-L1; therefore, we were unable to make a firm statement in this regard. Although previous works showed good recovery rates in cell lines and patients using Parsortix system [12,51,56], some methodological aspects can be impacting on the CTC enumeration in our cohort. For instance, we used CellSave Preservative tubes, which contain fixative reagents that can modify the deformability properties of the cells. Besides the staining protocol differs from previous publications [51] that described a higher detection rate using the Parsortix system in NSCLC.

Among the CTC population, we focused our attention on the PD-L1-positive subpopulation, which represents treatment targets. Although our data show that the determination of the PD-L1 status is feasible in CTCs from patients with NSCLC, we found that the PD-L1 status of CTCs does not correlate with the PD-L1 expression characterized in tissue samples or with the response to the treatment, regardless of the method employed. Despite the lack of prognostic value of PD-L1 expression on CTCs obtained using both approaches, most of the CTCs isolated using the Parsortix system were PD-L1-positive (100% of patients with CTCs), while only 20% of samples with CTCs contained PD-L1-positive CTCs using the

CellSearch[®] system. Thus, the epitope-independent-based Parsortix system showed a high recovery rate of PD-L1-positive CTCs, probably associated with a more mesenchymal phenotype of CTCs isolated with this EpCAM independent strategy. The use of CTCs and their potential to analyse PD-L1 expression has already been reported in patients with NSCLC [51,57–63], including the study by Janning, but their significance is not yet clear [64]. The lack of concordance and contradictory results between the presence of PD-L1 in tissue and the percentage of PD-L1-positive CTCs have also been reported [51,57,58,61,63,65]. Importantly, in most studies assessing PD-L1 expression in CTCs, different antibodies and CTC enrichment technologies have been used, which might partially explain the discrepancy.

Another important point is that CTCs originate from different tumour locations with different PD-L1 patterns [66], and tissue comparisons have been performed mainly with the primary tumour. Importantly, in our study, no association of PD-L1 expression on CTCs, such as prognostic or predictive biomarkers, was found. Despite the lack of clinical impact found for PD-L1-positive CTCs, the global CTC count at baseline determined using the CellSearch[®] system was significantly associated with PFS and OS, as previously described in patients treated with chemotherapy [67] and in patients receiving ICI treatment using the CellSearch[®] system or other technologies [13]. Our results revealed a greater effect of the main epithelial circulating population in patients with NSCLC, since the CTC count determined using a non-EpCAM-dependent strategy failed to show any association with the patients' outcomes.

Finally, our study represents a pioneering approach combining CTC count and cfDNA levels to predict the response of patients with NSCLC to first-line pembrolizumab treatment. We observed that patients with NSCLC presenting ≥ 1 CTC detected with the CellSearch[®] system and high levels of *hTERT* cfDNA at baseline had a significantly higher risk of disease progression during pembrolizumab treatment. Our results are consistent with a previous report focused on the prognostic role of these two easy-to-measure biomarkers in patients with metastatic NSCLC receiving nivolumab [15]. These results confirmed the value of combining different circulating biomarkers to reach a higher prognostic and predictive accuracy and better discriminate the patients who will benefit most from ICI treatment, in addition to PD-L1 status.

On the other hand, several limitations in our design should be considered, which precludes us from drawing solid conclusions. First, we used different antibodies to

analyse PD-L1 expression in CTCs and tumours, since the standard procedure was applied in tissue samples, while we used another antibody with CellSearch[®] and Parsortix. Second, we did not perform CTC monitoring during therapy in our NSCLC cohort, which could provide more valuable information. Third, *hTERT* amplification has been reported in different cancer types, including lung cancer [68,69]. Although the percentage of NSCLC patients with this alteration is very low (around 5–10% of lung cancer patients), we cannot exclude an overestimation of the cfDNA content in a low percentage of the cases analysed due to a potential amplification. In addition, the sample size of the combined cohort was relatively small.

5. Conclusions

In summary, the study served to establish the best strategy to monitor PD-L1 expression on CTCs from patients with advanced NSCLC. In addition, our results revealed that the combination of baseline CTCs and *hTERT* cfDNA levels is significantly associated with PFS and the response to pembrolizumab therapy in patients with metastatic NSCLC. Notably, using our approach, we were able to identify a subgroup of patients who were negative for CTCs, who presented low levels of *hTERT* cfDNA and who particularly benefited from the treatment. Early evaluation of the response to immunotherapy might enable clinicians to decide whether the clinical benefit is sufficient to continue treatment, avoiding unnecessary toxicities and costs.

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Conflict of interest

Jorge García-González reports personal fees from AstraZeneca, Boehringer Ingelheim, Novartis, Pierre Fabre, Rovi and Sanofi; and personal fees and nonfinancial support from Bristol-Myers Squibb, Lilly,

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Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version. RD-P takes responsibility for the integrity of the data and the accuracy of the data analysis. PM-M contributed to conceptualization, methodology, investigation, data curation, software and analysis of data. JG-G performed acquisition of data, investigation and data curation. LL-M performed acquisition of data, investigation and data curation. UA and SA performed acquisition of data. IA, MS-A and RML-L contributed to methodology. AA and AR-C contributed to methodology and data curation. AD-L contributed to methodology and investigation. LM-R and RL-L contributed to conceptualization, project administration and funding acquisition. RD-P contributed to study conception and design, analysis and interpretation of data, and supervision.

Peer Review

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Data accessibility

The data that support the findings of this study are available from the corresponding author (roberto.-diaz.pena@sergas.es) upon reasonable request.

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Supporting information

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

Fig. S1. *hTERT* cfDNA changes during Pembrolizumab therapy and their association with disease progression. (A) cfDNA levels at different time-points (baseline, 6 and 12 weeks); (B) cfDNA levels according to the response to therapy. cfDNA, circulating-free DNA.

Fig. S2. Immunofluorescence characterization of PD-L1 in cancer cell lines.

Fig. S3. Concordance analysis between the detection of CTCs (A) and CTCs PD-L1-positive (B) using the CellSearch® and Parsortix systems (Kappa test).

Fig. S4. Correlation of PD-L1 positivity between tumor tissues (by tumor proportion scores) and CTCs with the CellSearch® (A) and Parsortix systems (B).

Fig. S5. Objective response rate in patients with low cfDNA levels and undetectable CTCs (n = 12) versus patients with high cfDNA levels and undetectable CTCs or low cfDNA levels and detectable CTCs or high cfDNA levels and detectable CTCs (n = 18).

Table S1. ROC analysis to determine the value of *hTERT* cfDNA levels to discriminate progression or death.

Table S2. Circulating tumor cells enumeration and PD-L1 analysed using CellSearch® and Parsortix systems.

Table S3. Comparison of the CTCs levels according to the response to therapy.