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# Programmed Cell Death Ligand 1-Expressing Circulating Tumor Cells: A New Prognostic Biomarker in Non-Small Cell Lung Cancer

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**BACKGROUND:** In non-small cell lung cancer (NSCLC), analysis of programmed cell death ligand 1 (PD-L1) expression in circulating tumor cells (CTCs) is a potential alternative to overcome the problems linked to the tumor biopsy spatiotemporal heterogeneity. However, the prognostic significance of PD-L1-positive [PD-L1$^{(+)}$] CTCs remains controversial.

**METHODS:** We prospectively evaluated the correlation with clinicopathological variables and prognostic value of PD-L1$^{(+)}$ CTCs, detected with the FDA-cleared CellSearch® system, in 54 patients with advanced NSCLC.

**RESULTS:** We detected CTCs and PD-L1$^{(+)}$ CTCs in 43.4% and 9.4% of patients with NSCLC. PD-L1 expression concordance between tumor tissue and CTCs was low (54%). The presence of PD-L1$^{(+)}$ CTC correlated with the absence of gene alterations in tumor tissue and with poor prognosis-related biological variables (anemia, hyponatremia, increased lactate dehydrogenase). In univariate analysis, absence of gene alterations, number of metastatic sites, prior systemic therapies, and presence of CTCs and PD-L1$^{(+)}$ CTCs were associated with worse overall survival, whereas PD-L1 expression in tumor tissue was not. In multivariate analysis, squamous cell carcinoma histology, number of prior systemic treatments, and the presence of CTC were significantly associated with overall survival. Survival was worse in patients with PD-L1$^{(+)}$ CTCs than in patients with PD-L1-negative CTC or without any CTC.

**CONCLUSIONS:** Our study suggests that the presence of PD-L1$^{(+)}$ CTCs is associated with poor prognosis in patients with advanced NSCLC. Studies with larger samples are needed to confirm our results and to determine how PD-L1$^{(+)}$ CTC detection could help to predict the response or resistance to anti-PD-1/PD-L1 therapies.

Clinical trial registration NCT02866149

## Introduction

Lung cancer, mainly non-small cell lung cancer (NSCLC), is the leading cause of cancer death worldwide (1). Blockade of programmed cell death ligand 1 (PD-L1) interaction with its receptor programmed cell death 1 (PD-1) has revolutionized the treatment of NSCLC without oncogenic addiction, such as epidermal growth factor receptor (*EGFR*) alterations or anaplastic lymphoma kinase rearrangements. Nowadays, these immune checkpoint inhibitors are used alone as first- and second-line treatments of NSCLC (2), and also in association with chemotherapy in first-line settings (3).

Tumor PD-L1 expression is one of the most established predictive biomarkers of response to anti-PD-1/PD-L1 therapies, and is currently the only predictive biomarker used in clinical practice (4). However, >50% of patients with high PD-L1 expression in tumor do not benefit from first-line pembrolizumab (anti-PD-1 antibody) (2). Conversely, 10% of patients with a PD-L1-negative tumor respond to second-line anti-PD-1/PD-L1 agents (5). These findings may be explained by the spatiotemporal heterogeneity of PD-L1 expression in the tumor, and also by technical differences in the methods used for its detection (4).

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Circulating tumor cells (CTCs) might better reflect the tumor heterogeneity than tissue biopsies because they arise from different tumor sites (6). Furthermore, as CTCs are collected using a minimally invasive method (blood sampling), they can be analyzed longitudinally as liquid biopsies (7), and might provide information on the different mechanisms of treatment resistance. Several groups assessed PD-L1 expression on CTCs from patients with NSCLC using different methods, including the CellSearch® system (the only FDA-cleared CTC detection method) (8–11). However, they reported contradictory results in terms of concordance between PD-L1 expression in tumor tissue and in CTCs. Moreover, the presence of PD-L1-positive [PD-L1$^{(+)}$] CTCs at baseline does not predict the response to immune checkpoint inhibitors (12), and is associated with poorer prognosis (13, 14).

This study aim was to confirm the feasibility of PD-L1 assessment in CTCs detected with the CellSearch system, in a cohort of patients with advanced NSCLC, and to determine the concordance with PD-L1 expression status in tumor tissue biopsies. Moreover, the clinicopathological correlations and prognostic value of PD-L1$^{(+)}$ CTCs were prospectively investigated.

## Materials and Methods

### PATIENTS

This study was part of the ALCINA trial that assesses circulating biomarkers in different cancer types (NCT02866149). Patients >18 years of age with histologically confirmed stage III or IV NSCLC treated at the Montpellier Cancer Institute were prospectively enrolled between June 2016 and June 2018. Blood sampling was performed at diagnosis, before the first treatment ($n = 9$), or later, at progression, before the next therapeutic line ($n = 45$). All patients signed a written informed consent.

Patients were prospectively followed to determine progression-free survival (PFS) and overall survival (OS). Their baseline characteristics were extracted from their electronic health record.

### BLOOD SAMPLES AND CTC DETECTION

Blood was drawn from the arm vein in specific CellSave® tubes (Menarini, 10 mL) for CTC detection. Blood samples at room temperature were sent to the "Detection of Rare Human Circulating Cells–LCCRH" Laboratory at Montpellier University Medical Center, France, where they were processed immediately with the CellSearch system (Methods in the online Data Supplement).

PD-L1 expression in CTCs was evaluated with the antihuman B7-H1/PD-L1 fluorescein isothiocyanate-conjugated antibody (R&D System), the analytical sensitivity and specificity of which were demonstrated (15).

### STATISTICAL ANALYSIS

Frequencies and percentages were used for qualitative variables and means, medians, and ranges for continuous variables. For qualitative variables, percentages were calculated relative to the total population, excluding missing data. The Chi-square test and the Fischer's exact test were used to compare qualitative variables, and the Kruskal–Wallis test for quantitative variables.

Concordance between PD-L1 expression in the matched tumor tissue and CTCs was determined by calculating the clinical sensitivity, clinical specificity, positive, and negative predictive values, using the PD-L1 evaluation on tumor tissue as the gold standard.

The primary endpoints of the outcome analysis were PFS and OS. All survival times were calculated from the inclusion date and estimated with the Kaplan–Meier method and 95% confidence intervals (CI).

Univariate analysis was performed using the log rank test for qualitative variables and a Cox proportional hazards model for continuous variables. For the multivariate Cox models, variables were selected using a backward selection process, checking for cofounding effects at each step.

All statistical tests were bilateral and a $P < 0.05$ was considered significant. All statistical analyses were performed with STATA, v.16.0.

## Results

### DESCRIPTIVE ANALYSIS

*Baseline patients' characteristics.* Fifty-four patients with stage III–IV NSCLC were prospectively enrolled between June 2016 and June 2018. The patient characteristics are summarized in Table 1.

Their mean age was 64.5 years, 57.4% were men and 86% were smokers; 80% of them had a performance status score = 0 or 1. Most patients had stage IV NSCLC (94.4%); adenocarcinoma was the most frequent histological type (72.2%). Among the nonsquamous carcinomas (and squamous carcinomas in nonsmokers), 23 tumors (54.8%) had molecular alterations, including 8 targetable alterations [*EGFR* mutations and *c-ros oncogene 1 receptor tyrosine kinase* (*ROS1*) rearrangements].

The median time from diagnosis to inclusion was 18 months. Most patients (83.3%) had already had 1 or more treatment before inclusion: surgery (25.9%), radio-chemotherapy (7.4%), radiotherapy alone (24.1%), and at least 1 systemic treatment (68.5%).

*CTC detection, PD-L1 expression in tumor tissue samples and in CTCs.* Information on PD-L1 expression in tumor biopsies was available for 42/54 patients (77.8%). PD-L1

**Table 1. Patient characteristics at inclusion ($N = 54$).**

| Characteristics | *N* (%) |
|---|---|
| Age, years (mean, range) | 64.5 (34–84) |
| Sex | |
| Male | 31 (57.4) |
| Female | 23 (42.6) |
| Smoking status | |
| Never smoked | 7 (13.5) |
| Former smoker | 37 (71.1) |
| Current smoker | 8 (15.4) |
| NA | 2 |
| Performance status[a] | |
| 0 | 11 (22) |
| 1 | 29 (58) |
| 2 | 9 (18) |
| 3 | 1 (2) |
| NA | 4 |
| Histological type | |
| Adenocarcinoma | 39 (72.2) |
| SCC | 11 (20.4) |
| Others | 4 (7.4) |
| Molecular alteration (if applicable[b], $N = 42$) | |
| Targetable (EGFR/ROS1) | 8 (19.1) |
| Not targetable | 15 (35.7) |
| No | 19 (45.2) |
| Stage | |
| III | 3 (5.6) |
| IV | 51 (94.4) |
| Number of metastatic sites (mean, range) | 2.7 (0–6) |
| Prior treatment | |
| No | 9 (16.7) |
| Yes | 45 (83.3) |
| Surgery | 14 (25.9) |
| Radiotherapy or radio-chemotherapy | 17 (31.5) |
| Systemic treatment | 37 (68.5) |
| 1 line | 23 (42.6) |
| 2 lines | 7 (13) |
| 3 lines | 6 (11.1) |
| >3 lines | 1 (1.9) |

NA: not available; SCC: squamous cell carcinoma; EGFR, Epidermal growth factor receptor; ROS1, c-ros oncogene 1 receptor tyrosine kinase.
[a]Eastern Cooperative Oncology Group performance status score (on 5-point scale, with higher scores indicating increasing disability).
[b]Not applicable for SCC in smoker patients and neuroendocrine carcinoma.

expression was detected in at least 1% of tumor cells in 19/42 tumor biopsies (45.2%). PD-L1 expression in biopsies was assessed at a mean 5 months before inclusion. The most frequently used antibody was the clone E1L3N.

Among the 54 blood samples, one was not interpretable because of intensity saturation causing a reading problem in the CellTrack Analyzer. CTCs were detected in 23/53 samples (43.4%). The median CTC number per 7.5 mL of blood was 3 (range, 1–205). PD-L1$^{(+)}$ CTCs were detected in 5 blood samples (9.4%) (median number = 3; range, 1–4). In these 5 samples, the PD-L1$^{(+)}$ CTC subset represented 1.5 to 100% of all detected CTCs.

### PD-L1 EXPRESSION CONCORDANCE BETWEEN TUMOR BIOPSIES AND CTCS

Concordance between PD-L1 expression in tumor biopsies (≥1% of tumor cells) and CTCs could be analyzed in 41 patients (Table 2). Only 10.5% of patients with PD-L1-positive tumor biopsy had PD-L1$^{(+)}$ CTCs. Conversely, PD-L1$^{(+)}$ CTCs were detected in the blood sample of 9.1% of patients with PD-L1-negative biopsy. These results indicated a very low agreement between detection methods, with a concordance rate of 53.7%.

### CORRELATIONS OF THE PRESENCE OF CTC AND PD-L1$^{(+)}$ CTC WITH CLINICOPATHOLOGICAL VARIABLES

The presence of CTC was significantly correlated with lower body mass index (BMI) (22.5 vs 25.5, $P = 0.019$), higher number of metastatic sites (3.2 vs 2, $P = 0.003$), and lower lymphocyte levels (1.2 vs 1.6 G/L, $P = 0.037$) (Supplemental Table 1).

PD-L1$^{(+)}$ CTC detection was significantly correlated with absence of molecular alterations [no tumor in the PD-L1$^{(+)}$ CTC patient subgroup displayed molecular alterations compared with 23 tumors in the other 37 patients; 0% vs 62.2%, $P = 0.030$], lower concentrations of hemoglobin (11.1 vs 12.5 g/dL, $P = 0.028$) and sodium (136.4 vs 140.1 mmol/L, $P = 0.021$), and higher lactate dehydrogenase concentration (667.5 vs 294.2 IU/L, $P = 0.048$) (Supplemental Table 2).

All the correlations of the presence of CTCs and PD-L1$^{(+)}$ CTCs with clinicopathological variables are in Supplemental Tables 1 and 2, respectively.

The mean number of CTCs tended to be higher, but not significantly so, in patients with PD-L1$^{(+)}$ CTCs than in those without PD-L1$^{(+)}$ CTCs (52.2 vs 9.9, $P = 0.150$) (Supplemental Table 3). A logistic regression analysis found a positive, but not significant correlation between CTC number and presence of PD-L1$^{(+)}$ CTCs [odds ratio = 1.30 (0.82–2.05),

**Table 2.** Concordance between PD-L1 expression in tumor biopsy and CTCs ($N = 41$).

| | PD-L1 expression in tumor biopsy (≥1% of tumor cells) | | |
|---|---|---|---|
| | No | Yes | Total |
| | $N = 22$ | $N = 19$ | $N = 41$ |
| Presence of PD-L1$^{(+)}$ CTCs | | | |
| No | 20 (90.9%) | 17 (89.5%) | 37 (90.2%) |
| Yes | 2 (9.1%) | 2 (10.5%) | 4 (9.8%) |

Concordance rate = 53.7%.
Sensitivity = 10.5%, Specificity = 90.9%.
Positive predictive value = 50.0%, Negative predictive value = 54.0%.

$P = 0.267$], possibly due to the low sample size (Supplemental Table 4).

### CORRELATION OF CTCS AND PD-L1$^{(+)}$ CTCS WITH CLINICAL OUTCOMES

At the cutoff date (December 15, 2020), the median follow-up was 44.9 months (95% CI: 33.0–52.4). Because of the low number of detected CTCs, a threshold of 1 CTC was used for survival analyses. Cutoffs of 2 or 5 CTCs implied comparing groups with nonhomogeneous sizes (Supplemental Table 5).

*CTC presence correlates with progression-free survival.* In univariate analysis, the presence of CTCs was significantly associated with worse PFS [median PFS: 2.6 vs 8.3 months, Hazard Ratio, HR = 2.27 (1.28–4.04), $P = 0.006$] (Fig. 1, A).

The presence of PD-L1$^{(+)}$ CTCs (Fig. 2, A) did not change PFS [HR = 1.89 (0.72–4.95), $P = 0.200$]. Conversely, PFS was shorter in patients with PD-L1-negative CTCs compared with patients without CTCs [HR = 2.43 (1.30–4.55), $P = 0.005$]. PD-L1 expression in tumor biopsies was not correlated with PFS [HR = 0.67 (0.36–1.25), $P = 0.204$] (Supplemental Table 6).

Clinicopathological features with a weak correlation with PFS ($P < 0.25$) are summarized in Supplemental Table 6. Molecular alterations, particularly presence of a targetable alteration, were significantly associated with longer PFS [HR = 0.50 (0.26–0.95), $P = 0.004$ and HR = 0.31 (0.12–0.81), $P = 0.006$, respectively]. Conversely, PFS was significantly worse in former smokers than in patients without smoking history [HR = 3.22 (1.31–7.92), $P = 0.017$].

To assess the prognostic value of the presence of CTCs and PD-L1$^{(+)}$ CTCs, 3 multivariate models were built: (*a*) Model 1 [patients with PD-L1$^{(-)}$ CTCs and PD-L1$^{(+)}$ CTCs vs patients without CTC]; (*b*) Model 2 (all patients with at least one CTC vs patients without CTC); and (*c*) Model 3 [patients with PD-L1$^{(+)}$ CTC vs all other patients].

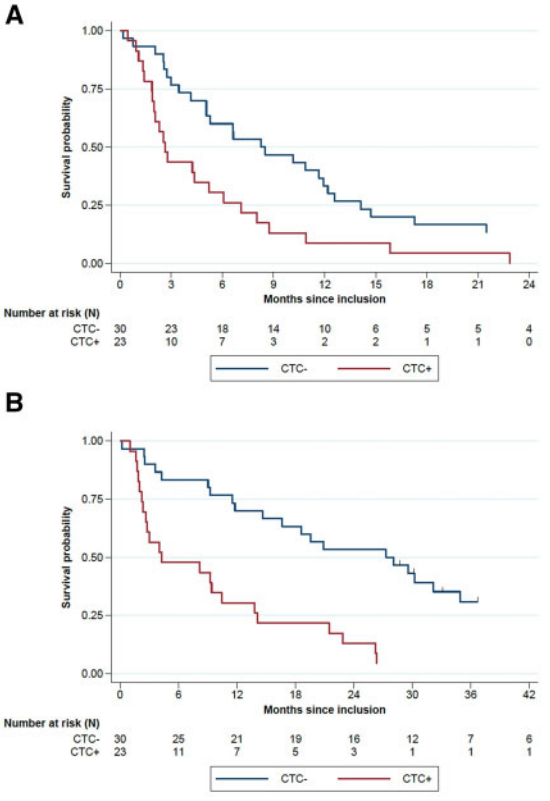


**Fig. 1.** Progression-free survival (A) and overall survival (B) in patients with and without circulating tumor cells. CTC+, patients with at least 1 circulating tumor cell; CTC−, patients without any circulating tumor cell.

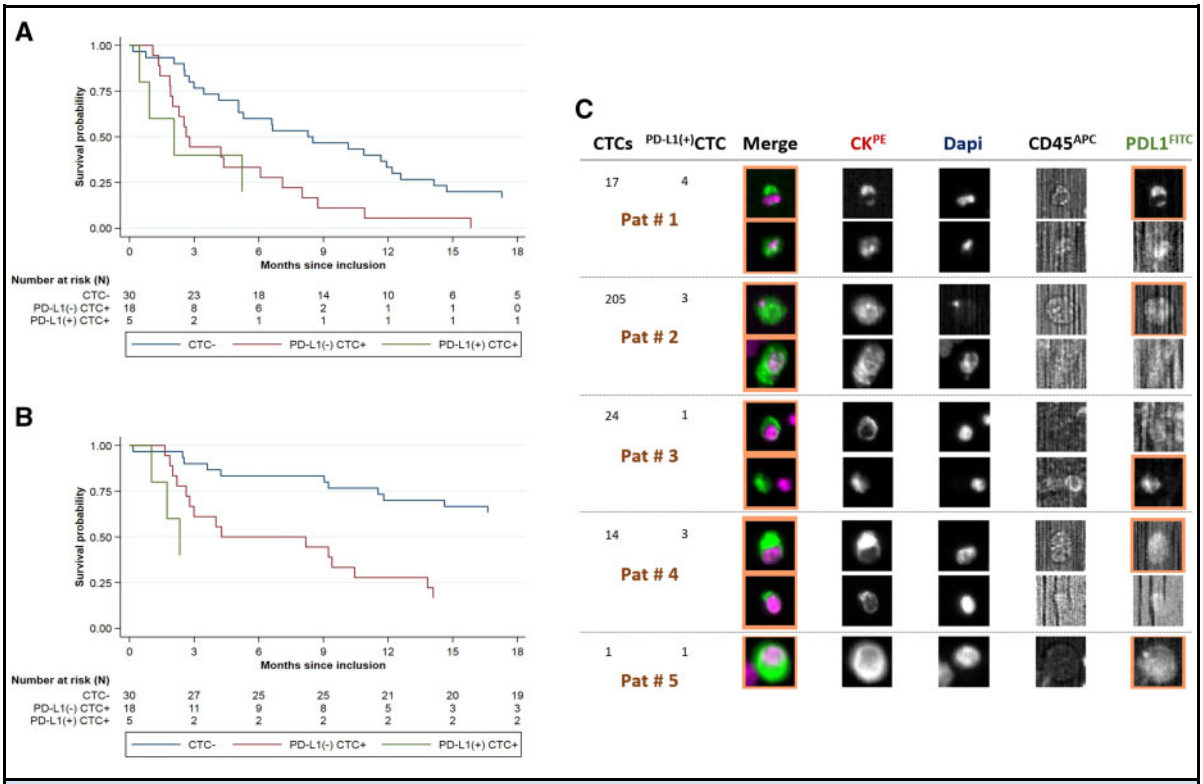


**Fig. 2.** Progression-free survival (A) and overall survival (B) according to the circulating tumor cell PD-L1 status. (C), Representative images of PD-L1$^{(+)}$ CTCs and PD-L1$^{(-)}$ CTCs detected in the blood samples of the 5 positive metastatic non-small cell lung cancer patients for PD-L1$^{(+)}$ CTCs using the CellSearch system. Column 1 represents the total number of CTCs and column 2 the number of CTCs expressing PD-L1. Column 3 represents the merge picture, which is the superposition of the CK$^{PE}$ + DAPI, CD45$^{APC}$ photos–columns 4-6. The PD-L1$^{(+)}$ CTCs are highlighted with an orange frame in column 7. PD-L1$^{(+)}$–CTC, patients with at least 1 circulating tumor cell that expressed PD-L1; PD-L1$^{(-)}$–CTC, patients with at least 1 circulating tumor cell, but without PD-L1 expression; CTC$^{(-)}$, patients without any circulating tumor cell; EpCAM, epithelial cell adhesion molecule; CK$^{PE}$, cytokeratin-phycoerythrin; DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate.

All variables significantly correlated with the presence of CTCs or PD-L1$^{(+)}$ CTCs in the correlation analysis (Supplemental Tables 1 and 2) and those that showed a moderate association with PFS in univariate analysis ($P < 0.25$) were tested in each model. At the end of this selection process, each model included hemoglobin concentration (continuous variable).

These models showed that PFS was shorter in patients with at least 1 CTC than in those without CTC [Model 2: HR = 2.03 (1.12–3.69), $P = 0.022$]. Conversely, the presence of PD-L1$^{(+)}$ CTCs did not influence PFS in models 1 and 3 (Table 3).

*Overall survival is worse in patients with PD-L1$^{(+)}$ CTCs* The presence of CTCs was significantly associated with shorter OS compared with no CTC detection [median OS: 4.3 vs 27.3 months respectively, HR = 3.06 (1.65–5.70), $P < 0.001$] (Fig. 1, B). OS was significantly worse in patients with PD-L1$^{(-)}$ CTCs and particularly in patients with PD-L1$^{(+)}$ CTCs compared with patients without CTCs [HR = 2.93 (1.51–5.68) and 3.63 (1.33–9.91), respectively, $P = 0.002$] (Fig. 2, B). Conversely, PD-L1 expression in the tumor biopsy was not associated with OS [HR = 0.75 (0.38–1.48), $P = 0.407$].

Four other variables significantly affected OS in univariate analysis: presence of a molecular alteration [HR = 0.41 (0.21–0.81), $P = 0.011$], number of metastatic sites [HR = 1.25 (1.02–1.54), $P = 0.037$], more than 2 lines of previous systemic therapy [HR = 4.8 (1.73–13.3), $P = 0.044$], and previous platinum-based doublet chemotherapy [HR = 2.07 (1.10–3.91), $P = 0.020$] (Supplemental Table 7).

Three multivariate models were built using the same method as for the PFS analysis. All models finally included the number of prior systemic therapy lines

**Table 3.** Adjusted hazards ratios for the variables associated with progression-free survival. Multivariate Cox model.

| | Model 1 (*N* = 51) | | Model 2 (*N* = 51) | | Model 3 (*N* = 51) | |
|---|---|---|---|---|---|---|
| **Variable** | **HR** | **95% CI** | **HR** | **95%CI** | **HR** | **95%CI** |
| PD-L1 status in CTCs | | ***P* = 0.032** | | | | |
| $CTC^{(-)}$ | 1.00 | Ref | | | | |
| $PD\text{-}L1^{(-)}$ CTCs | 2.48 | [1.28; 4.79] | | | | |
| $PD\text{-}L1^{(+)}$ CTCs | 1.24 | [0.44; 3.45] | | | | |
| CTC presence | | | | ***P* = 0.022** | | |
| No | | | 1.00 | Ref | | |
| Yes | | | 2.03 | [1.12; 3.69] | | |
| $PD\text{-}L1^{(+)}$ CTC presence | | | | | | ***P* = 0.974** |
| No | | | | | 1.00 | Ref |
| Yes | | | | | 0.98 | [0.37; 2.65] |
| Hemoglobin (g/dL) | | ***P* = 0.017** | | ***P* = 0.040** | | ***P* = 0.020** |
| Increase of 1 unit | 0.78 | [0.63; 0.96] | 0.81 | [0.66; 1.00] | 0.78 | [0.63; 0.96] |

**Table 4.** Adjusted hazards ratios for the variables associated with overall survival. Multivariate Cox model.

| | Model 1 (*N* = 53) | | Model 2 (*N* = 53) | | Model 3 (*N* = 53) | |
|---|---|---|---|---|---|---|
| **Variable** | **HR** | **95% CI** | **HR** | **95% CI** | **HR** | **95% CI** |
| PD-L1 status in CTCs | | ***P* = 0.001** | | | | |
| $CTC^{(-)}$ | 1.00 | Ref. | | | | |
| $PD\text{-}L1^{(-)}$ CTCs | 3.70 | [1.67; 8.17] | | | | |
| $PD\text{-}L1^{(+)}$ CTCs | 5.51 | [1.93; 15.73] | | | | |
| CTC presence | | | | ***P* = 0.001** | | |
| No | | | 1.00 | Ref. | | |
| Yes | | | 4.11 | [1.99; 8.50] | | |
| $PD\text{-}L1^{(+)}$ CTC presence | | | | | | ***P* = 0.024** |
| No | | | | | 1.00 | Ref. |
| Yes | | | | | 3.69 | [1.36; 9.98] |
| Number of previous systemic therapy lines | | ***P* = 0.016** | | ***P* = 0.016** | | ***P* = 0.003** |
| 0 | 1.00 | Ref. | 1.00 | Ref. | 1.00 | Ref. |
| 1–2 | 2.44 | [1.12; 5.33] | 2.48 | [1.14; 5.42] | 2.07 | [0.94; 4.55] |
| >2 | 4.33 | [1.38; 13.62] | 3.97 | [1.30; 12.10] | 7.30 | [2.40; 22.24] |
| Histological type | | ***P* = 0.007** | | ***P* = 0.007** | | ***P* = 0.021** |
| Adenocarcinoma | 1.00 | Ref. | 1.00 | Ref. | 1.00 | Ref. |
| SCC | 3.62 | [1.66; 7.91] | 3.63 | [1.66; 7.94] | 2.89 | [1.37; 6.09] |
| Others | 2.74 | [0.73; 10.26] | 2.59 | [0.70; 9.59] | 2.90 | [0.76; 11.13] |

SCC: squamous cell carcinoma.

and tumor histological type (Table 4). The presence of CTCs was significantly associated with worse OS [Model 2: HR = 4.11 (1.99–8.50), $P = 0.001$]. Unlike the PFS results, OS was worse in patients with PD-L1$^{(+)}$ CTCs compared with patients with PD-L1$^{(-)}$ CTCs and without CTCs in model 1 [HR of PD-L1$^{(+)}$ CTC presence vs CTC absence = 5.51 (1.93–15.73) and HR of PD-L1$^{(-)}$ CTC presence vs CTC absence = 3.70 (1.67–8.17), $P = 0.001$], and when compared with patients without PD-L1$^{(+)}$ CTCs in model 3 [HR = 3.69 (1.36–9.99), $P = 0.024$].

Finally, high number of previous systemic treatments and squamous cell carcinoma histological type were significantly correlated with worse OS in all models.

Although the presence of molecular alterations was positively correlated with PFS and OS in univariate analysis, this variable was not included in the multivariate models because it concerned only a subset of the target population. Multivariate analyses for PFS and OS including this variable are in Supplemental Tables 8 and 9, respectively.

## Discussion

CTC immunocytological characterization, as a liquid biopsy, might better reflect NSCLC heterogeneity than tumor biopsy analysis (10). Here, we confirmed that PD-L1 expression status can be investigated in CTCs from patients with advanced NSCLC using the FDA-cleared CellSearch system. We detected CTCs and PD-L1$^{(+)}$ CTCs in 43.4% and 9.4% of patients, respectively. The concordance rate of PD-L1 expression in matched tumor biopsy and CTCs was only 53.7%. The presence of CTCs (CTC $\geq$1) was significantly associated with worse PFS and OS. Despite the low number of patients with PD-L1$^{(+)}$ CTCs, their detection was significantly correlated with worse OS, and tended to be associated with shorter PFS. Conversely, PD-L1 expression in the tumor biopsy did not have any prognostic impact.

We found that 43.4% of patients had at least 1 detectable CTC, in line with other studies using the CellSearch technology (i.e., 40 to 60% of patients) (16, 17). The negative prognostic value of CTC count in advanced NSCLC has been demonstrated by different studies using the CellSearch system, with a threshold of 5 or 2 CTCs per 7.5 mL of blood (18). Here, we found that the presence of one CTC had a prognostic impact, which is relevant considering CTC scarcity in NSCLC. The correlation between CTC detection and number of metastatic sites has been well described (16, 19), whereas its association with lower concentration of circulating lymphocytes and lower BMI has been less frequently reported.

The presence of CTCs correlates with lower concentrations of T lymphocytes and natural killer cells in peripheral blood of patients with NSCLC (20). These previous findings and our results suggest a close relationship between altered immune surveillance and CTC presence. In breast cancer, presence of CTCs has been associated with an increase of peripheral CD95(FAS)-positive T-helper cells (21). Similarly, another study recently demonstrated that CTC detection in NSCLC was linked to upregulation of several inhibitory checkpoint receptors (T-cell immunoglobulin mucin-3, PD-1, cytotoxic T-lymphocyte antigen-4, and lymphocyte-activation gene-3) on tumor-infiltrating lymphocytes (22). Finally, CTC association with lower BMI has been observed also in metastatic breast cancer (23). However, we cannot exclude a confounding effect, because cachexia is more likely to occur in patients with NSCLC and high tumor burden (24).

Some studies reported a positive correlation between presence of *EGFR* alterations and CTC detection (25). In our study, CTC detection rate was not different in the subgroup of patients with targetable alterations (7 *EGFR* alterations and one *ROS1* rearrangement), in agreement with a recent meta-analysis (18). However, the low number of patients in this subgroup precludes any definitive conclusion.

In 2015, our group detected PD-L1 expression on CTCs from patients with metastatic breast cancer (26). In 2016, PD-L1 expression on CTCs from 19 patients with metastatic NSCLC was assessed, highlighting the challenge of detecting PD-L1 on CTCs, because many immune cells express this marker (27). Subsequently, the feasibility and clinical significance of PD-L1 detection on CTCs in NSCLC was investigated (8). Here, we detected at least one PD-L1$^{(+)}$ CTC in 9.4% of patients. Considering all previously published studies, the detection rate ranges from 7.5% (28) to 100% (29). This large variability may be mainly related to differences in detection methods, such as CTC enrichment technique [antigen-dependent (11, 30) or antigen-independent methods (28)], antibodies against PD-L1, and analytical thresholds for single-cell PD-L1 positivity. Only one study used the CellSearch system for CTC detection in 24 patients with metastatic NSCLC, and found CTCs and PD-L1$^{(+)}$ CTCs in 83% and 79% of samples, respectively (11). The high detection rate in this study, compared with our findings, could be explained by the fact that all patients were heavily pretreated (100% received at least 1 prior line of systemic therapy vs 68.5% in our study). Additional studies are needed to better explain these discrepancies and to determine PD-L1$^{(+)}$ CTC rate in advanced NSCLC. Mostly, it will be crucial to assess their clinical relevance.

We found a significant correlation between PD-L1$^{(+)}$ CTC detection and absence of molecular alterations. Especially, none of the patients in the PD-L1$^{(+)}$ CTC group had targetable alterations in the tumor

tissue. Similarly, another study reported that the number of PD-L1$^{(+)}$ CTCs was significantly higher in patients with adenocarcinoma without EGFR mutation than with EGFR mutation ($n = 67$ patients in total) (31). This is in line with the observation that PD-L1 positivity on tissue is more frequent in NSCLC with wild type EGFR (32), probably because tumors with oncogene addiction, associated with low rate of tobacco-induced carcinogenesis, show a weak immunogenicity (33). On the other hand, the correlation with several biological variables related to poor prognosis and advanced disease (anemia, hyponatremia, high activity of lactate dehydrogenase) is not surprising because PD-L1$^{(+)}$ CTC detection is associated with higher NSCLC stage (29).

To our knowledge, this is the first study to assess the prognostic value (OS/PFS) of PD-L1$^{(+)}$ CTC detection in advanced NSCLC using the FDA-cleared CellSearch technology. PFS tended to be shorter in patients with PD-L1$^{(+)}$ CTCs, but did not reach significance, probably due to small sample size. Conversely, PD-L1 expression on tumor tissue, which is a very debated prognostic factor in NSCLC, was not associated with PFS and OS. Some publications also found a negative prognostic impact of PD-L1$^{(+)}$ CTCs in NSCLC (13, 14), whereas other studies did not report any significant prognostic value of this CTC subpopulation (34). Again, these discrepancies may be related to differences in CTC enrichment and detection methods as well in the choice of the anti-PD-L1 antibodies.

PD-L1 upregulation is one of the mechanisms through which tumor cells escape the immune system (35). In different cancer types, including lung cancer (36), PD-L1 upregulation has been associated with epithelial-to-mesenchymal transition (EMT). Most PD-L1$^{(+)}$ CTCs display an unusual elongated morphology, suggesting partial EMT (11). Furthermore, mesenchymal markers and PD-L1 are frequently coexpressed in CTCs from patients with NSCLC (29, 37). It has been hypothesized that tumor cells that undergo EMT gain migratory properties and stem cell-like features, and consequently, could become more aggressive (38). Interestingly, these CTCs can still express EpCAM, which is crucial for metastasis-competent CTCs (39). Indeed, EpCAM may not be completely lost during EMT, and in our study, this marker was used for CTC capture. We can hypothesize that EMT and PD-L1 upregulation on EpCAM$^{(+)}$ CTCs are 2 mechanisms that cooperate to promote tumor cell dissemination. However, we found a trend to higher CTC count in patients with PD-L1$^{(+)}$ CTCs. We could not assess the independent prognostic value of PD-L1$^{(+)}$ CTCs in model 1 due to the small sample size [$n = 5$ patients with detectable PD-L1$^{(+)}$ CTCs]. Thus, we cannot rule out that the prognostic impact of PD-L1$^{(+)}$ CTC detection could be linked to the overall higher number of detected CTCs, a feature frequently associated with worse prognosis.

The concordance analysis may have been skewed by the temporal heterogeneity of PD-L1 expression analysis because the mean interval between tissue and blood sampling was 5 months. However, other studies did not find any correlation between PD-L1 expression on tumor tissue and on CTCs (29, 31, 40), even when blood and tumor sampling were done at the same time (29, 40), supporting the spatial heterogeneity of this biomarker (40–42). In 2 cases, we found that PD-L1 was expressed on CTCs but not on the tumor biopsy, as already reported (29, 43). This feature may be explained by PD-L1 expression at metastatic sites from which CTCs originated, because PD-L1 expression is usually higher at distant sites than at the primary site (44). We recently reported similar data on patients with breast cancer where only PD-L1 detection in CTCs, but not in the tumor, had a clinical relevance (15). These findings suggest that CTCs, which may reveal PD-L1 spatial heterogeneity, are a complementary tool for PD-L1 expression detection with a clear clinical relevance in NSCLC.

Finally, several studies suggest that the persistence or increase of PD-L1$^{(+)}$ CTCs during immunotherapy may mirror a mechanism of therapy escape (11, 40). This could not be evaluated here because immune checkpoint inhibitors had only restricted indications when the project was started. Our ongoing study (NCT04025541), in which PD-L1$^{(+)}$ CTCs are monitored during anti-PD-1/PD-L1 therapy, will help to determine whether this CTC subpopulation can predict the response or resistance to these immunotherapies.

In summary, our data confirm the feasibility of PD-L1 detection on CTCs in patients with advanced NSCLC, using the CellSearch technology, and suggest a negative prognostic impact of the PD-L1$^{(+)}$ CTC subpopulation. The weak concordance between PD-L1 expression on the tumor tissue and CTCs and the finding that only PD-L1 expression on CTCs predicted the clinical outcome suggest that tissue biopsy and CTCs are 2 complementary tools. Larger studies are needed to confirm our findings and to determine how PD-L1$^{(+)}$ CTCs, as liquid biopsy, could help to predict the response or resistance to anti-PD-1/PD-L1 therapies with the aim of proposing personalized treatments to patients with NSCLC.

## Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

**Nonstandard Abbreviations:** PD-L1, programmed cell death ligand 1; NSCLC, non-small cell lung cancer; CTC, circulating tumor cell; OS, overall survival; PD-1, programmed cell death 1; EGFR, epidermal growth factor receptor; PFS, progression-free survival; EpCAM,

epithelial cell adhesion molecule; CK, cytokeratin; BMI, body mass index; EMT, epithelial-to-mesenchymal transition; FITC, fluorescein isothiocyanate

**Human Genes:** *EGFR*, epidermal growth factor receptor; *ROS1*, c-ros oncogene 1 receptor tyrosine kinase

**Ethics Approval and Consent to Participate:** All patients provided their written informed consent and were prospectively included in the ethics committee-approved ALCINA study (NCT02866149) that investigates the feasibility of analysis of circulating tumor markers in blood in various early- or advanced-stage malignancies.

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