# Evaluation of PD-L1/PD-1 on circulating tumor cells in patients with advanced non-small cell lung cancer

*Ther Adv Med Oncol*

2018, Vol. 10: 1–11

DOI: 10.1177/1758834017750121



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***Abstract***

**Background:** Circulating tumor cells (CTCs) could escape from the immune system through the programmed death-ligand 1 (PD-L1)/programmed cell death protein 1 (PD-1) axis leading to the development of metastasis. The current study investigated the expression of PD-1/PD-L1 on CTCs isolated from non-small cell lung cancer (NSCLC) patients treated with chemotherapy.

**Patients and methods:** CTCs were isolated from 30 chemo-naïve stage IV NSCLC patients before and after front-line chemotherapy using the ISET filtration platform. CTCs were detected by Giemsa and immunofluorescence (IF) staining. Samples were analyzed with the ARIOL system.

**Results:** Giemsa staining revealed that 28 (93.3%) out of 30 and 9 (81.8%) out of 11 patients had detectable CTCs at baseline and after the third chemotherapy cycle, respectively. Cytokeratin (CK)+/CD45- CTCs by IF could be detected in 17 of 30 (56.7%) patients at baseline and in 8 of 11 (72.7%) after the third chemotherapy cycle. Spearman analysis revealed a significant correlation ($p = 0.001$) between Giemsa-positive and IF-positive (CK+/CD45-) CTCs. At baseline, PD-1 and PD-L1 expression was observed in 53% and in 47% CK-positive patients, respectively. After the third treatment cycle the corresponding numbers were 13% and 63% respectively. Median progression-free survival (PFS) was significantly shorter in patients with >3 PD-1(+) CTCs at baseline compared with those with <3 PD-1(+) CTCs ($p = 0.022$) as well as in patients with >1 Giemsa-positive tumor cells ($p = 0.025$).

**Conclusion:** PD-1(+) and PD-L1(+) CTCs could be detected before and after front-line chemotherapy in patients with metastatic NSCLC. The presence of high PD-1(+) CTC numbers before treatment is associated with a poor patient clinical outcome.

***Keywords:*** CTCs, NSCLC, PD-L1/PD-1

Received: 21 September 2017; revised manuscript accepted: 1 December 2017.

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## Introduction

Programmed cell death protein 1 (PD-1) is an inhibitory immune checkpoint and has been identified as an indicator of exhausted CD8+ effector T-cells in chronic diseases including cancer. Programmed death-ligand 1 (PD-L1) is expressed on tumor cells and its interaction with PD-1 on the surface of immune cells regulates negatively the immune system. The blockade of the PD-1/PD-L1 interaction can partially restore T-cell function,[1] allowing the antitumor efficacy of effector CD8+ lymphocytes.[2,3] High levels of PD-L1 on tumor cells has been correlated with poor prognosis in distinct tumor types.[4]

Dissemination of tumor cells (disseminated tumor cells; DTCs) in the bone marrow or in the blood (circulating tumor cells; CTCs) is an obligatory step toward the development of metastatic disease.[5–8]

The detection of CTCs has been associated with unfavorable clinical outcome in cancer patients.[9–14] Indeed, the detection of CTCs in patients with non-small cell lung cancer (NSCLC) before treatment has been associated with reduced progression-free survival (PFS) and overall survival (OS).[15] However, the detection rate of CTCs in patients with NSCLC considerably varies depending on the isolation methods,[11,16] suggesting a heterogeneity of CTCs. Indeed, it has been reported that the detection rate of CTCs in patients with advanced NSCLC was higher using the ISET system compared with the CellSearch platform;[11,16] ISET-isolated CTCs from patients with NSCLC have been shown to bear important chromosomal instability and genetic rearrangements.[17,18]

Antibodies (Abs) against PD-1/PD-L1 are a new class of immune checkpoint inhibitors with documented antitumor activity through the blockage of the interactions between inhibitory receptors expressed on T-cells and their ligands on tumor cells. A number of studies have investigated whether the efficacy of antibodies against immune check point inhibitors correlates to PD-L1 expression on tumor cells. Although high expression of PD-L1 on tumor cells has been proposed as a reliable predictor of efficacy for anti-PD-L1/PD-1 Abs, this is not the case for patients whose tumors present a low PD-L1 expression.[19,20] The difficulty to more precisely define the value of PD-L1 expression on tumor cells might be related to tumor cell heterogeneity which has been, recently, emerged as an important factor leading to treatment resistance.[21]

Previous studies from our group had focused on the use of CTCs as biosource to define real-time biomarkers useful for disease prognostication[22] or selection of targeted treatment.[23–25] The expression of PD-L1 in CTCs has been addressed in a limited number of studies so far. Indeed, positive CTCs have been identified in patients with breast and head/neck cancer and the presence of PD-L1(+) CTCs has been shown to be associated with patients' clinical outcomes.[26–29] There are also very few data in the literature concerning the expression of PD-L1 in CTCs in patients with NSCLC.[27,30,31] Particularly, the study by Schehr and colleagues[31] was a methodological approach for the detection of PD-L1 expressing-CTCs. Furthermore, Nicolazzo and colleagues[30] evaluated the efficacy of nivolumab in a small group ($n = 24$) of patients with NSCLC according to the PD-L1 expression on CTCs; the authors reported that the presence PD-L1(+) CTCs was associated with a poor patient outcome.

In the current study, we sought to investigate the expression of PD-1 and PD-L1 on CTCs in newly diagnosed chemotherapy-naïve patients with advanced stage NSCLC during front-line chemotherapy using the ISET platform.

## Materials and methods

### *Cancer cell lines*

The following lung cancer cell lines were used in control experiments, in order to define the pattern of PD-1 and PD-L1 expression for subsequent evaluation of clinical samples: H460 cell line (large cell lung cancer), H1299 (lymph node metastasis of a lung adenocarcinoma), HCC827 (adenocarcinoma lung cancer) and SKMES (squamous cell carcinoma). All cell lines were obtained from ATCC (American Type Culture Collection, USA). The H1299 cell line was cultured in 1:1 Dulbecco's Modified Eagle Medium (GIBCO-BRL Co, MD, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO-BRL), and 50 mg/ml penicillin/streptomycin. The SKMES cell line was cultured in MEM-alpha (GIBCO-BRL) with 10% FBS and 50 mg/ml penicillin/streptomycin. The HCC827 cell line was cultured in RPMI (GIBCO-BRL) plus 10% FBS and penicillin/streptomycin. Finally, the H460 cell line was cultured in RPMI, 5% sodium pyruvate and 10% FBS and penicillin/streptomycin. Sub-cultivation of all cell lines was performed with 0.25% trypsin (GIBCO-BRL) and 5 mm EDTA (GIBCO-BRL). Cells were maintained in a humidified atmosphere of 5% $CO_2$ in air. All experiments were performed during the logarithmic growth phase of the cells. For spiking experiments various dilutions (10 cells/ml, 100 cells/ml and 1000 cells/ml of blood) of cells from the four cell lines were spiked in 10 ml of blood obtained from healthy blood volunteers in order to mimic the CTC microenvironment in our control samples.

### *Patients' blood samples*

Peripheral blood (10 ml in EDTA) was obtained from 30 chemotherapy-naïve patients with metastatic NSCLC before the administration of front-line chemotherapy (baseline sample); a second blood sample was also obtained in 11 of them after the third chemotherapy cycle at the time of assessment of treatment efficacy. In the remaining

patients, a second blood sample was not available either because of early disease progression before assessment of treatment efficacy or early death or, finally, for technical reasons. Blood samples were obtained at the middle of vein puncture after the first 5 ml of blood were discarded in order to avoid contamination of the blood sample with epithelial cells from the skin during sample collection. The protocol has been approved by the Ethics and Scientific Committees of our institution and all patients and healthy blood donors gave their informed consent to participate in the study.

### *ISET isolation system*

CTCs were isolated using the ISET platform according to the manufacturer instructions. Briefly, 10 ml of peripheral blood were diluted in 1:10 ISET buffer (Rarecells, Paris, France) for 10 min at room temperature (RT), and 10 ml of the diluted sample was filtered using depression tab adjusted at 10 KPa. The membrane was dried for 2 h at RT and stored at −20°C. Each spot of the membrane was used for identification of CTCs after staining with May-Grünwald Giemsa and for immunostaining and fluorescent microscopy.

### *May-Grünwald Giemsa staining*

CTCs captured in the ISET filters were stained according to a validated protocol provided by the manufacturer.[32,33] Briefly, spots were stained using pure May-Grünwald Giemsa solution (Sigma Life Science, Taufkirchen, Germany) for 3 min and then incubated in diluted 1:2 May-Grünwald Giemsa for 3 min. Subsequently, samples were placed in Giemsa solution (Sigma Life Science, Germany) diluted 1/10 and incubated for 15 min. Spots were, then, rinsed with PBS and dried at RT. Samples were analyzed with bright field microspore by two experienced pathologists (EL and AK).

### *Immunofluorescence staining*

CTCs from patients, as well as cancer cells from spiking experiments, were analyzed for the expression of PD-L1 and PD-1 using double and triple IF staining with the corresponding antibodies. Triple IF staining for cytokeratin (CK)/PD-1/CD45 and CK/PD-L1/CD45 was performed in cancer patients who harvested PD-1 or PD-L1 positive CTCs with double IF in order to confirm that the cells characterized as CTCs were not hematopoietic cells. The samples were, subsequently, evaluated using the ARIOL system. This platform can automatically identify the mean fluorescence intensity per pixel for each isolated tumor cell.

For IF staining, spots were incubated with PBS for 5 min and then the cells were permeabilized with 2% Triton for 10 min. After 1 h blocking with PBS/10% FBS, cells were incubated either with anti-PD-1/FITC (Clone EH 12.2H7, Biolegend, San Diego, USA) or anti-PD-L1 (clone B7-H1/PD-L1/CD274; Novus Biologicals, Abingdon, UK) antibodies. For the detection of PD-L1 the samples were further incubated with Alexa 555 anti-rabbit antibody (Invitrogen, Carlsbad, CA, USA).

For the detection of CKs two different antibodies were used as a cocktail: the clone A45-B/B3 anti-mouse Ab recognizing the CKs 8/18/19 (Micromet Munich, Germany) and an anti-mouse Ab against CK7 (clone RCK105; Abcam, Cambridge, UK). CK7 was used in order to increase the recovery rate of CTCs since this cytokeratin is highly expressed in NSCLC tumor cells;[34] Consequently, in case of PD-L1/CK/CD45 staining, Alexa 488 (Invitrogen) anti-mouse was used as a secondary antibody for the identification of CK. In case of PD-1/CK/CD45 staining, the secondary antibody for CK was the Alexa 555 (Invitrogen) anti-mouse because PD-1 was already conjugated with FITC. Consequently, anti-CD45 antibody conjugated with Alexa 647 (clone HI30; Novus Biologicals) was also added in order to exclude the hematopoietic nature of the cells.

Positive controls were used in each experiment using the above cell lines spiked in healthy volunteers' blood while negative controls were prepared by omitting the corresponding primary antibodies and incubating the cells with the matching immunoglobulin (Ig)G isotype bound to the corresponding fluorochrome. The cytomorphological criteria described by Meng were used for the characterization of a cell as a CTC.[35]

## Results

### *PD-1 and PD-L1 expression in cell line tumor cells*

The mean intensity staining for PD-1 and PD-L1 of the four NSCLC cell lines is presented in Figure 1 (A, B) and Suppl. Figure 2. The highest mean intensity per pixel of PD-1 and PD-L1 staining was observed in HCC827 cells [181.4 ± 26.3 and

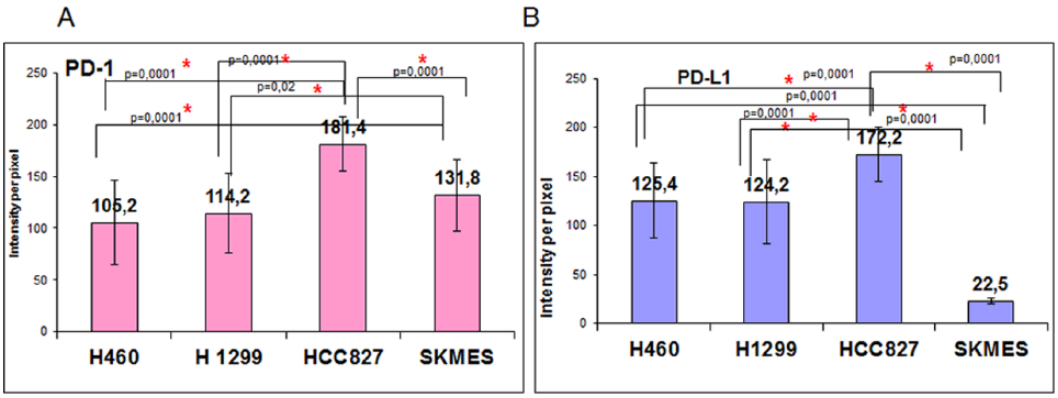



**Figure 1.** Quantification of PD-1 and PD-L1 in control cell lines.
(A) Mean intensity per pixel of PD-1 expression in H460, H1299, HCC827 and SKMES cell lines, automatically quantified by ARIOL system.
(B) Mean intensity per pixel of PD-L1 expression in H460, H1299, HCC827 and SKMES cell lines, automatically quantified by ARIOL system.
PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1.

172.2 ± 27.7 respectively; Figure 1(A, B)] whereas, the lowest intensity of PD-1 staining was observed in the H460 cells (105.2 ± 40.8) and for PD-L1 in the squamous cell line SKMES (22.5 ± 3). The mean intensity of PD-1 staining was significantly different among the cell lines [Figure 1(A)]. Based on these findings, CTCs with expression intensity higher than the observed in H460 and SKMES cells were considered as positive for PD-1 and PD-L1 expression, respectively.

### *Detection of CTCs in NSCLC patients*

The presence of CTCs was evaluated with Giemsa and IF. The morphologic analysis [Figure 2(A)] of the captured CTCs after May -Grünwald Giemsa staining led to the following observations: (i) CTCs presented a high nuclear-to-cytoplasmic (N/C) ratio and exhibited prodigious polymorphism, which translated in morphologic phenotypes that ranged from epithelioid to mesenchymal; (ii), there were significant variations in cell size, shape and nuclear quality; (iii) nuclei were, usually, hyperchromatic with sizes that alternated from bland small cells (a, b, c, d, e) to large cells (f) exhibiting one or more small nucleoli or even macronucleoli. Cells could be identified as a single CTC (a) or in clusters (g, h). Giemsa staining revealed CTCs in 28 of 30 (93.3%) studied patients at baseline and in 9 of 11 (81.8%) patients studied after the third chemotherapy cycle [Figure 2(B)]. The median number of detected CTCs was 5 CTCs/ml of blood (range, 0–23 CTCs/ml of blood).

Using IF staining [Figure 2(C), Suppl. Figure 1], CTCs could be detected in 17 of 30 (56.7%) patients at baseline and in 8 of 11 (72.7%) after the third treatment cycle [Figure 2(B)]. The concordance between the two detection methods at baseline and after the third treatment cycle was 63.3% and 67%, respectively. A statistical significant correlation was observed in both time points between CK-positive and Giemsa-positive tumor cells (Spearman test: $p = 0.001$).

### *Detection of PD-L1(+) CTCs in NSCLC patients*

In the whole group of patients, the rate of detection of PD-L1(+) CTCs was 26.7% (8 out of 30) at baseline while after the third cycle this percentage increased to 45.5% (5 out of 11) [$p = 0.096$; Figure 3(A)]. The mean number of CK+/PD-L1+ CTCs was 1 CTC/ml of blood (range; 1–8 CTCs/ml) at baseline. After the third cycle of treatment, the mean number of CK+/PD-L1+ CTCs was 1.2 (range; 1–4 CTCs/ml). Among the CK-positive patients, PD-L1(+) CTCs were identified in 47% (8/17) at baseline and in 63% (5/8) CK-positive patients after the third cycle of treatment. All the cells that were characterized as double positive were found as single CTCs in the blood stream.

Among the total examined CTCs, PD-L1 expression was observed in 15% of the isolated tumor cells at baseline, while after treatment the corresponding frequency was 80%. The mean intensity of the PD-L1 expression on CTCs according to ARIOL system was 128.905 ± 0.3. In addition, 20% (6/30) and 18.2% (2/11) patients

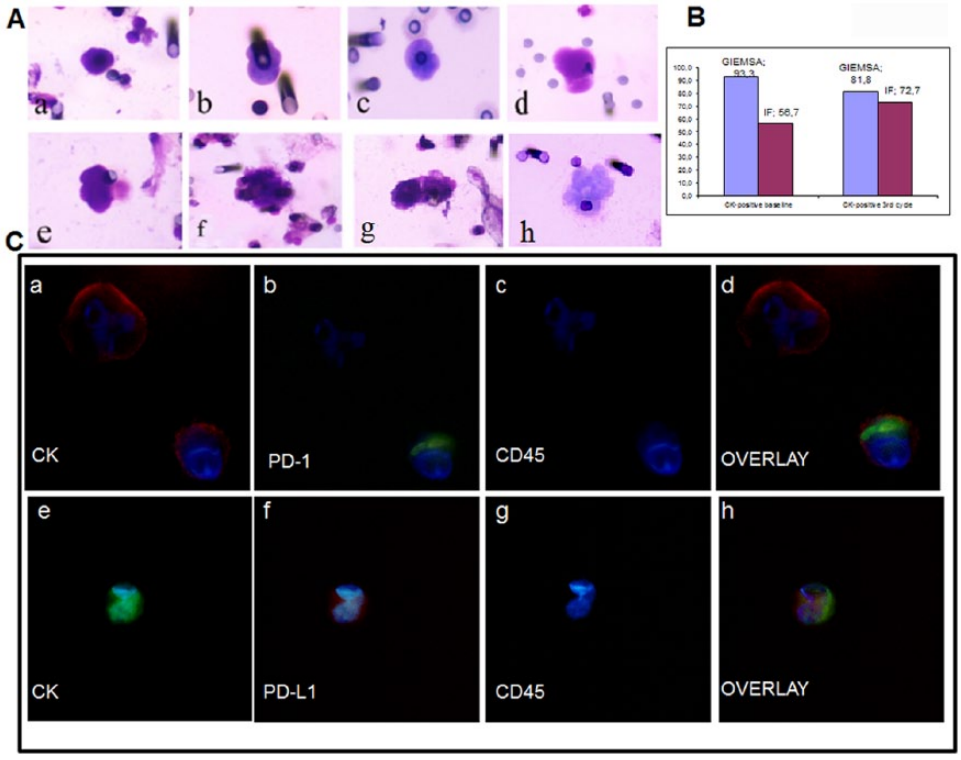



**Figure 2.** CTCs in NSCLC patients.
(A) Representative Giemsa staining in patients' samples.
(B) Percentage of NSCLC patients with CTCs at baseline and after the third cycle as evaluated by IF and Giemsa staining.
(C) (a–d): Representative ARIOL Images (magnification ×40) of CTCs stained with PD-1/CK/CD45. In the same patient, both [PD-1+(green)/CK+(orange)/CD45-(far red)]] and (PD-1-/CK+/CD45-) phenotypes were observed. (e–h): Representative ARIOL Images (magnification ×40) of CTCs stained for PD-L1 [(orange)/CK (green)/CD45(far red)] antibodies (magnification ×40).
CK, cytokeratin; CTC, circulating tumor cell; IF, immunofluorescence; NSCLC, non-small cell lung cancer; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1.

harvested PD-L1(−) CTCs at baseline and after the third chemotherapy cycle, respectively [Figure 3(B)]. The absolute number of CTCs per patient is shown in Table 1. Only two patients at baseline and one at the third cycle harvested simultaneously two different CTC phenotypes [PD-L1(+) and PD-L1(−)].

### *Detection of PD-1(+) CTCs in NSCLC patients*

In the whole group of patients, the rate of detection of CK(+)/PD1(+) CTCs was 30% (9/30) and 9.1% (1/11) at baseline and after the third cycle of treatment, respectively [$p = 0.785$; Figure 3(C)]. Similarly, among the CK-positive patients at baseline, CK(+)/PD1(+) CTCs were detected in 9 out of 17 (53%) and in 1 of 8 (13%) patients at baseline and after 3 cycles of treatment, respectively [$p = 0.301$; Figure 3(B)]. All the PD-1 positive CTCs were found as single cells.

Among the total examined CTCs, PD-1 expression was observed in 66.7% of the cells at baseline, while after the third cycle only 25% of the CTCs were PD-1(+). The mean intensity of PD-1 on CTCs was $117.4 \pm 2.16$. Furthermore, PD-1(−) CTCs were detectable in 16.7% of patients at baseline and in 45.5% after the third chemotherapy cycle [Figure 3(D)]. Table 1 presents the absolute number of CTCs per phenotype both at baseline and after the third cycle of treatment. It is interesting that only one patient out of 30 harvested simultaneously two different phenotypes [PD-1(+) and PD-1(−)] of CTCs.

### *Clinical outcome according to the presence of PD-L1(+) and PD-1(+) CTCs*

After a median follow-up period of 5 months (range, 1–13), patients harvesting >1 CTCs/ml

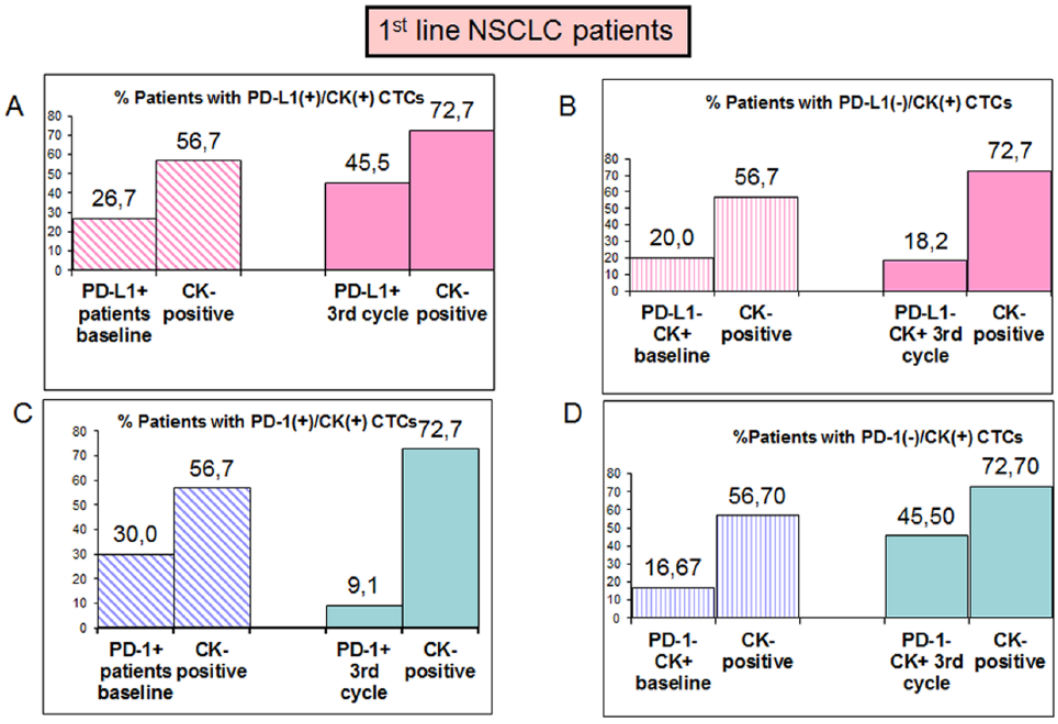



**Figure 3.** Expression of PD-1 and PD-L1 NSCLC patients.
(A) Percentage of patients expressing CK(+)/PD-L1(+)/CD45(-) cells at baseline and after the 3rd cycle of treatment as compared to the CK-positive group of patients.
(B) Percentage of patients expressing CK(+)/PD-L1(-)/CD45(-) at baseline and after the 3rd cycle of treatment as compared to the CK-positive group of patients.
(C) Percentage of patients expressing CK(+)/PD-1(+)/CD45(-) cells at baseline and after the 3rd cycle of treatment as compared to the CK-positive group of patients.
(D) Percentage of patients expressing CK(+)/PD-1(-)/CD45(-) cells at baseline and after the 3rd cycle of treatment as compared to the CK-positive group of patients.

of blood ($n$ = 25), as defined by Giemsa, had a significantly shorter PFS compared with patients with 0–1 CTCs/ml ($n$ = 5) of blood [6.5 months *versus* 2.9 months, respectively; hazard ratio (HR) = 1.104, $p$ = 0.025; Figure 4(A)].

Similarly, patients ($n$ = 2) with >3 CK(+)/PD-1(+)/ml of blood had a significantly shorter PFS compared with patients with <3 CK(+)/PD-1(+) CTCs/ ml of blood ($n$ = 28) [0.5 months *versus* 3.9 months, respectively; HR = 1.14, $p$ = 0.022; Figure 4(B)]. There was no significant difference in terms of OS among the two groups of patients.

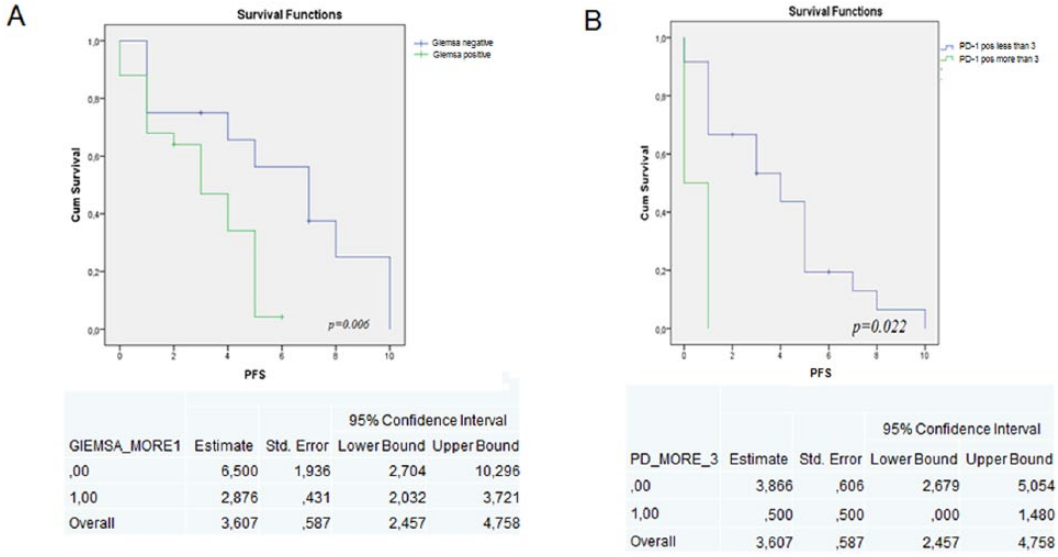
## Discussion

Metastasis is the leading cause of cancer-related death; however, the mechanisms that govern this process are still under investigation. There is substantial evidence that CTCs are involved in the metastatic process and, thus, their appropriate targeting could be emerged as an important tool to prevent their dissemination and development of metastasis.[23–25] PD-1/PD-L1 crosstalk between cancer and immune cells is well-studied. PD-L1 expression on tumor cells has been correlated with poor prognosis in different tumor types.[4,19] There are a number of studies evaluating the PD-1/PD-L1 expression on primary tumor cells as a predictive biomarker for the efficacy of immune checkpoint inhibitors and, therefore, as a tool for the selection of candidate patients for immunotherapy. However, most of these studies evaluated PD-L1 expression on an archival tumor sample which has been proposed as a major obstacle for the safe determination of PD-L1 expression on the tumor cells. Consequently, there is a great need for a re-biopsy in order to real-time evaluate the PD-L1 status which, in daily clinical practice, is difficult to accomplish. In this context, the presence of viable CTCs, could be a valuable source to longitudinally evaluate the PD-L1 expression on tumor cells during the different clinical phases of

**Table 1.** Phenotype of CTCs at baseline and after the third chemotherapy cycle.

| Baseline samples | | | | | | Third cycle samples | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Patients** | **GIEMSA** | **PD-1+/CK+** | **PD-1-/CK+** | **PD-L1+/CK+** | **PD-L1-/CK+** | **Patients** | **GIEMSA** | **PD-1+/CK+** | **PD-1-/CK+** | **PD-L1+/CK+** | **PD-L1-/CK+** |
| 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 |
| 2 | 9 | 0 | 0 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 0 |
| 3 | 5 | 0 | 2 | 0 | 0 | 3 | 5 | 0 | 1 | 0 | 0 |
| 4 | 21 | 3 | 0 | 8 | 4 | 4 | 1 | 0 | 0 | 0 | 0 |
| 5 | 1 | 0 | 0 | 0 | 0 | 5 | 10 | 0 | 0 | 4 | 0 |
| 6 | 8 | 2 | 3 | 1 | 0 | 6 | 3 | 0 | 1 | 0 | 0 |
| 7 | 1 | 1 | 0 | 0 | 0 | 7 | 6 | 0 | 2 | 0 | 1 |
| 8 | 3 | 0 | 0 | 0 | 0 | 8 | 1 | 0 | 0 | 0 | 0 |
| 9 | 5 | 0 | 0 | 1 | 0 | 9 | 6 | 0 | 1 | 1 | 0 |
| 10 | 3 | 1 | 0 | 1 | 0 | 10 | 1 | 2 | 0 | 1 | 1 |
| 11 | 9 | 0 | 0 | 0 | 0 | 11 | 5 | 0 | 0 | 0 | 0 |
| 12 | 6 | 2 | 0 | 0 | 0 | | | | | | |
| 13 | 7 | 1 | 0 | 0 | 2 | | | | | | |
| 14 | 8 | 0 | 0 | 0 | 2 | | | | | | |
| 15 | 4 | 0 | 0 | 0 | 0 | | | | | | |
| 16 | 1 | 0 | 0 | 0 | 0 | | | | | | |
| 17 | 0 | 0 | 0 | 0 | 0 | | | | | | |
| 18 | 3 | 0 | 0 | 0 | 0 | | | | | | |
| 19 | 5 | 0 | 0 | 0 | 0 | | | | | | |
| 20 | 5 | 0 | 2 | 0 | 2 | | | | | | |
| 21 | 2 | 0 | 2 | 0 | 0 | | | | | | |
| 22 | 8 | 0 | 0 | 0 | 0 | | | | | | |
| 23 | 2 | 1 | 0 | 0 | 0 | | | | | | |
| 24 | 20 | 8 | 0 | 1 | 9 | | | | | | |
| 25 | 4 | 0 | 1 | 1 | 0 | | | | | | |
| 26 | 5 | 0 | 0 | 1 | 0 | | | | | | |
| 27 | 2 | 0 | 0 | 0 | 0 | | | | | | |
| 28 | 2 | 0 | 0 | 0 | 0 | | | | | | |
| 29 | 8 | 0 | 0 | 0 | 0 | | | | | | |
| 30 | 23 | 1 | 0 | 0 | 24 | | | | | | |

CK, cytokeratin; CTC, circulating tumor cell; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1.




| | | | 95% Confidence Interval | |
|---|---|---|---|---|
| GIEMSA_MORE1 | Estimate | Std. Error | Lower Bound | Upper Bound |
| ,00 | 6,500 | 1,936 | 2,704 | 10,296 |
| 1,00 | 2,876 | ,431 | 2,032 | 3,721 |
| Overall | 3,607 | ,587 | 2,457 | 4,758 |

| | | | 95% Confidence Interval | |
|---|---|---|---|---|
| PD_MORE_3 | Estimate | Std. Error | Lower Bound | Upper Bound |
| ,00 | 3,866 | ,606 | 2,679 | 5,054 |
| 1,00 | ,500 | ,500 | ,000 | 1,480 |
| Overall | 3,607 | ,587 | 2,457 | 4,758 |

**Figure 4.** CTCs and clinical outcome.
(A) Patients with >1 Giemsa(+) CTC at baseline experienced lower PFS.
(B) Patients with >3 PD-1(+) CTCs at baseline experienced lower PFS.
CTC, circulating tumor cell; PD-1, programmed cell death protein 1; PFS, progression-free survival.

the disease. Indeed, recent studies have reported the expression of PD-L1 on CTCs isolated from breast, bladder, head and neck and NSCLC.[26–28,30,31,36]

The current study evaluated both the expression of PD-1 and PD-L1 on CTCs isolated from newly diagnosed and chemotherapy-naïve patients with stage IV NSCLC. The CTCs were isolated using the ISET platform[16–18,37] and their detection was performed using both May -Grünwald Giemsa staining as well as triple immunofluorescence staining using antibodies against CK, PD-1or PD-L1 and CD45. It is interesting to note that both methods of detection gave practically similar results as assessed by Spearmen analysis ($p = 0.001$). Nevertheless, Giemsa was able to detect a higher number of patients with detectable CTCs, irrespectively of the treatment status and this could be, probably attributed to the fact that many CTCs in patients with NSCLC are under epithelial to mesenchymal transition and they express low level of CK.[11,38,39]

The intensity of PD-L1 and PD-1 was first evaluated on tumor cell lines in order to define whether the expression of these molecules is homogeneous irrespectively of the tumor histology. Indeed, the quantification of the intensity staining revealed significant differences between the different cell lines (Figure 1). This quantification gave a more objective cutoff in order to characterize a cell as PD-L1(+) or PD-1(+). The current study clearly indicated the presence of both PD-L1(+) and PD-1(+) CTCs in patients with untreated NSCLC. Moreover, our findings indicate, for the first time, that in some patients, CTCs may also express the PD-1 receptor both before and after chemotherapy, implying a bilateral crosstalk between immune cells and CTCs.

An interesting observation of the present study was that after three cycles of chemotherapy, the detection of CK(+)/PD-1(+) CTCs was reduced while, the detection of CK(+)/PD-L1(+) CTCs was increased; these findings are in agreement with the knowledge that treatment can influence the frequency of CTCs[22,24] which further supports the hypothesis that chemotherapy might preferentially eliminate a subpopulation of CTCs [PD-1(+)]. Interestingly, the study by Nicolazzo and colleagues[30] also reported an increase of the PD-L1(+)CTCs after 3 months of treatment with nivolumab in patients with NSCLC, suggesting that PD-L1(+) tumor cells are resistant to both chemotherapy and targeted therapy.[30]

Despite the fact that the number of studied patients was low, the detection of CTCs was associated with patients' clinical outcomes. Indeed, the detection of >1 CTCs/ml of blood by

Giemsa at baseline was significantly associated with decreased PFS ($p = 0.025$). Moreover, it is interesting to note that the detection of >3 CK(+)/PD-1(+) CTCs/ml of blood by IF at baseline, was also associated with shorter PFS ($p = 0.022$) compared with patients with <3 CK(+)/PD-1(+) CTCs/ml of blood. This finding raises important questions regarding the role of PD-1 expression on CTCs during immune recognition and whether antibodies against this molecule also target CTCs. Alternatively, we cannot exclude that PD-1 expression on CTCs may be involved in an interactive crosstalk between immune cells and CTCs.[40–42] However, it should be mentioned that any correlation between CTC phenotype and clinical outcome is only exploratory due to the observational nature of the study.

In conclusion, the current study demonstrates the presence of CTCs in patients with recurrent/metastatic NSCLC expressing both PD-1 and PD-L1 at baseline and after 3 cycles of chemotherapy, suggesting that these cells could be a useful biologic material in order to investigate biomarkers associated with the efficacy of immune checkpoints inhibitors.

## Conclusions

PD-1 and PD-L1 molecules are effective targets for the treatment of NSCLC patients. This study evaluated the expression of both molecules in patients' CTCs and the presented data demonstrate that CTCs are an easily accessible biologic material compared with repeated biopsies. Our results demonstrated that both PD-1 and PD-L1 molecules are expressed in newly diagnosed chemotherapy-naïve patients with NSCLC suggesting a crosstalk between immune cells and CTCs in the blood steam. Patients harboring >3 PD-1(+) CTCs had a significantly shorter PFS.

## Acknowledgements

Authors acknowledge the support of this work partly by the Hellenic Oncology Research Group (HORG), the Greek General Secretary of Research and Technology grant Oncoseed PN 3175, and the Cretan Association for Biomedical Research. We would like to sincerely thank the scientific secretary Vasso Athanasaki for her attentive editing of this manuscript.

GK participated in the design, coordination, supervision, interpretation, statistical analysis of the data and the preparation of the manuscript; KV performed staining experiments and in the study design; DA performed immunofluorescence experiments and ISET isolations; PK and MT provided technical assistance; EL and AK evaluated Giemsa staining; IM participated in IF evaluation; VG provided general support, participated in the study design and in manuscript preparation; AK participated in the design, coordination and supervision of the study and provided the clinical samples.

## Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

## Conflict of interest statement

The authors declare that there is no conflict of interest.

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