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Early View

Original article

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High Sensitivity of PD-L1 analysis from pleural effusion in non-small cell lung cancer

Short title: PD-L1 analysis from pleural effusion in lung cancer

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Author Contributions

L. Hagmeyer: Substantial contributions to conception and design, analysis and interpretation of data, drafting the article, and finalizing the version to be published taking responsibility for the integrity of the work as a whole, from inception to published article.

Stephan Schäfer: Acquisition of data, analysis and interpretation of data, revising the article critically for important intellectual content, final approval of the version to be published

Marianne Engels: Acquisition of data, analysis and interpretation of data, revising the article critically for important intellectual content, final approval of the version to be published

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Abbreviations:

EMA	European Medicines Agency
FDA	United States Food and Drug Administration
mNSCLC	metastatic non-small cell lung cancer
NSCLC	non-small cell lung cancer
PD-1	programmed cell death protein 1
PD-L1	programmed cell death protein ligand 1
PE	pleural effusion
TPS	tumor proportion score

Abstract

Background:

PD-1/PD-L1 immune checkpoint inhibitors have been approved for monotherapy of metastatic non-small cell lung cancer (mNSCLC) depending on tumor cells' PD-L1 expression. Pleural effusion (PE) is common in mNSCLC. The significance of immunocytochemistry PD-L1 analysis from PE samples is unclear.

Aim of the study:

To analyze the sensitivity regarding immunocytochemistry PD-L1 analysis of PE in NSCLC as compared to immunohistochemistry of pleural biopsies.

Patients and Methods:

50 consecutive subjects (17 female, median age 72.5, 7 never-smokers) were enrolled in this prospective controlled two-center study. Inclusion criteria were PE, suspected or known lung cancer, indication for pleural puncture and thoracoscopy, written informed consent. Immunocytochemistry and immunohistochemistry PD-L1 analyses were performed with the Dako-PDL1-IHC-22C3pharmDx assay. Analysis for sensitivity, specificity, positive (PPV) and negative predictive value (NPV) was performed for PD-L1 detection from PE.

Results:

50 subjects underwent pleural puncture and thoracoscopy. Pathologic diagnoses were lung cancer (48), lymphoma (1), mesothelioma (1). Sensitivity, specificity, positive-predictive-value and negative-predictive-value of PD-L1-testing with expression ≥50% defined as positive were 100% (95% confidence interval 46-100%), 63%(36-84%),

45%(18-75%), 100%(66-100%), and with expression ≥1% defined as positive 86%(56-97%), 43%(12-80%), 75%(47-92%), 60%(17-93%).

Conclusion:

PD-L1 analysis in tumor-positive PE samples shows a very high sensitivity and negative-predictive-value, especially regarding PD-L1 expression levels ≥50% (European Medicines Agency approval). Negative results are reliable and help in the decision against a first-line checkpoint inhibitor monotherapy. However, a 1% cut-off level (United States Food and Drug Administration approval) leads to a markedly lower negatve-predictive-value, making other invasive procedures necessary. (NCT02855281)

Introduction

The programmed cell death protein 1 (PD-1) physiologically acts as an immune checkpoint receptor, enabling self-tolerance by T-cells in normal tissue. Unbound PD-1 allows the normal immune response by T-cells to occur. Binding of PD-1 to the ligands PD-L1 and PD-L2 suppresses the immune response. PD-L1 expression on tumor cells leads to activation of PD-1 and suppression of cytotoxic T-cell activity. The T-cell tolerance allows the tumor cells to avoid recognition and elimination by the immune system [1].

Early studies showed that immune checkpoint inhibitors had positive therapeutic effects in non-small cell lung cancer (NSCLC) patients with detectable PD-L1 expression [2]. Positive results could be demonstrated in treatment-naïve as well as in previously treated advanced NSCLC. There is probably a correlation between tumor PD-L1 expression and improved antitumor activity [3].

Pembrolizumab, Nivolumab and Atezolizumab are the available PD-1/PD-L1 immune checkpoint inhibitors which have been approved by the European Medicines Agency (EMA) and United States Food and Drug Administration (FDA) for the treatment of metastatic NSCLC (mNSCLC) stage IV according to the Union for international cancer control. Remarkably, EMA and FDA approvals differ for certain indications (Appendix Table 1). Major differences are

a) the approval of Pembrolizumab for 1st-line monotherapy in mNSCLC expressing PD-L1 with a tumor proportion score (TPS) ≥50% according to EMA and with a TPS ≥1% according to FDA, respectively.

b) the approval of Atezolizumab solely by the FDA for 1st-line monotherapy in mNSCLC with ≥50% of tumor cells expressing PD-L1.

Lung cancer is the second most common cancer and is the primary cause of cancer-related death in both men and women in the United States [4]. Currently, 80% of patients with lung cancer are given a diagnosis of primary NSCLC. Malignant pleural effusion (PE) is a common complication of advanced lung cancer. The presence of malignant PE indicates a poorer prognosis for patients with lung cancer and reduces their quality of life. PE is a convenient clinical sample with important clinical diagnostic significance. It may be an alternative source providing useful information about the neoplasm's biology in terms of molecular genetic and immunopathologic profile.

This prospective diagnostic pilot study was conducted to analyze the sensitivity regarding immunocytochemistry PD-L1 analysis of PE in NSCLC patients compared to the reference standard of PD-L1 immunohistochemistry.

The primary analysis goal was to test whether immunocytochemistry analysis of PD-L1 from PE has a relevant diagnostic value as compared to the immunohistochemistry analysis of pleural biopsies as the reference standard. In this analysis the specific approval status of checkpoint inhibitors in the EMA and the FDA region was considered.

Material and methods

Patients

At two tertiary care centers patients presenting with PE and suspected or known underlying lung cancer with indication for pleural puncture and thoracoscopy between October 2016 and November 2018 were considered for potential study inclusion. Exclusion criteria comprised given contraindications to undergo thoracoscopy [5] and any medical, psychological or other condition impairing the patient's ability to provide informed consent. Histopathological data from bronchoscopic tissue samples were available in 40 cases.

Study design

The trial was a prospective controlled pilot two-center study. The study was approved by the Ethics Commission of Cologne University's Faculty of Medicine and registered on www.clinicaltrials.gov (NCT02855281). All patients gave their written informed consent before study-specific data collection. All interventions were undertaken as part of clinical routine.

Sampling pleural cytology

The patients underwent ultrasound guided puncturing of the pleural cavity at the location of effusion. The excess pleural fluid was removed and collected for later cytologic analysis by an independent pathology department (SS, ME, RB).

Sampling pleural histology

Histological pleural samples were obtained by pleural biopsy, primarily applying an awake single-incision medical video-assisted rigid thoracoscopy (Storz, Germany) under analgosedation using Midazolam, Disoprivane and Pethidine. If this approach

was not feasible, the patient underwent a surgical 2-4-ports video-assisted thoracoscopy under general anesthesia. In either case, at least nine biopsies from the parietal pleura were taken. Where feasible, biopsies were sampled from the dorsocaudal, dorsal/dorsoapical, ventral and diaphragmatic parietal pleura.

Pathology

All samples were sent in for routine pathological analyses. Microscope slides from paraffin embedded tissue and cell blocks from cytocentrifugation samples were analyzed. In cases where malignant tumor cells could be detected, subsequent immunocytochemistry or immunohistochemistry PD-L1 analysis was performed with the Dako PD-L1 IHC 22C3 pharmDx assay (Agilent, Santa Clara, CA, USA). PD-L1 expression was quantified by a 5-level Score [6-7]. For the assessment of potentially therapy-relevant PD-L1 expression levels, two different threshold levels of PD-L1 expression were considered as per the differing approval status (TPS ≥50% and TPS≥1%).

Regarding detection of malignancy and PD-L1-positivity, results were defined as negative where the tests were not feasible or in case of inconclusive findings.

Statistical analysis

Results of continuous variables are expressed as median and quartiles 1 and 3. The number of PD-L1-positive and PD-L1-negative cases for each sample type were used for calculation of sensitivity and specificity.

Secondary analyses comprised intra-individual comparative descriptive analyses of all pathological results from all patients to assess correlation between immunocytochemistry and immunohistochemistry results on a qualitative level. All

analyses were performed using IBM SPSS Statistics™ for Windows (version 26.0.; IBM Corp., Armonk, N.Y., USA).

Results

Patients and diagnostic procedures

Fifty patients were enrolled in this study. Their anthropometric data and smoking status are shown in Table 1. In 41 out of the enrolled 50 cases the disease was classified as NSCLC (Adenocarcinoma n=33; squamous cell carcinoma n=7; large cell neuroendocrine carcinoma n=1) and 9 patients showed other histologic malignancy types (small cell lung cancer n=4; mixed carcinoma n=3; non-Hodgkin-lymphoma n=1; sarcomatoid mesothelioma n=1).

Pleural puncture as well as thoracoscopy were performed in all fifty patients. No relevant periinterventional complications have been documented. PD-L1 analysis of PE and pleural tissue was indicated in all samples with evidence of tumor cells, excluding cases with small-cell lung cancer. Mainly due to insufficient sample material (low tumor cell count), especially in pleural effusion, PD-L1 analysis could actually be performed in 25 (PE) and 35 (pleural tissue) cases (Figure 1).

Pleural histology, cytology and PD-L1 status

The pathological evaluation of PE led to inconclusive results in 7 cases, only describing cells with “suspicious” properties. The majority of cases where PD-L1 analysis was feasible showed PD-L1 expression with a TPS ≥1%. In PE 13 of 25 cases (52%, 95% confidence interval 34-70%) were PD-L1-positive at a TPS ≥50%, while in pleural tissue 8 of 35 cases (23%, 95% confidence interval 12-39%) were PD-L1-positive (Table 2). A detailed breakdown of all cases regarding the detection of malignant tumor cells as well as PD-L1 status is given in the appendix, Table 2.

Estimated sensitivity, specificity, positive and negative predictive value of tumor cell detection according to immunocytochemistry analysis of PE compared to immunohistochemistry analysis of pleural biopsies were 83% (95% Confidence interval (95%CI) 67-89%), 70% (95%CI 35-92%), 92% (95%CI 76-98%), 50% (95%CI 24-76%).

Sensitivity and specificity of PD-L1 detection in pleural effusion based on all cases with indication for PD-L1 analysis

Based on all cases where PD-L1 analysis was indicated, the immunocytochemistry analysis of PE was compared with the immunohistochemistry analysis of pleural tissue (appendix, table 3). Two different alternatives were calculated:

- PD-L1 expression with a TPS ≥50% was defined as PD-L1-positive

- PD-L1 expression with a TPS ≥1% was defined as PD-L1-positive

Estimated sensitivity and specificity of PD-L1 detection (PD-L1 expression with a TPS ≥50% defined as positive) according to immunocytochemistry analysis of PE compared to immunohistochemistry analysis of pleural biopsies were 71% (95%CI 30-95%) and 71% (95%CI 49-87%). The positive predictive value was 42% (95%CI 16-71%) and the negative predictive value was 89% (95%CI 65-98%). The positive and the negative likelihood ratio was 1.98 (95%CI 0.92-4.26) and 0.46 (95%CI 0.20-1.05), respectively.

Estimated sensitivity and specificity of PD-L1 detection (PD-L1 expression with a TPS ≥1% defined as positive) according to immunocytochemistry analysis of PE compared to immunohistochemistry analysis of pleura biopsies were 71% (95%CI 44-89%) and 64% (95%CI 36-86%). The positive predictive value was 71% (95%CI 44-89%) and

negative predictive value was 64% (95%CI 36-86%). The positive and the negative likelihood ratio was 1.98 (95%CI 0.92-4.26) and 0.46 (95%CI 0.20-1.05), respectively.

Sensitivity and specificity of PD-L1 detection in pleural effusion based on all cases with successful PD-L1 analysis

Based on all successful PD-L1 analyses (i.e. excluding samples with insufficient material for analysis or technical issues), the immunocytochemistry analysis of PE was compared with the immunohistochemistry analysis of pleural tissue (table 3). Two different alternatives were calculated:

- PD-L1 expression with a TPS ≥50% was defined as PD-L1-positive.

- PD-L1 expression with a TPS ≥1% was defined as PD-L1-positive.

Estimated sensitivity, specificity, positive and negative predictive value and likelihood ratios of PD-L1 detection according to immunocytochemistry analysis of PE compared to immunohistochemistry analysis of pleural biopsies are given in table 3.

Sensitivity and negative predictive value were very high and the negative likelihood ratio was very good with a PD-L1 expression TPS ≥50% defined as positive. For this cut-off value negative test results are robust and reliable as they may allow to dispense from more invasive diagnostic procedures.

On the other hand, sensitivity and negative predictive value were lower with a PD-L1 expression TPS ≥1% defined as positive. For this cut-off value the test results may not be robust enough in the clinical decision-making process for or against a single agent first-line checkpoint inhibitor therapy.

For both cut-off values specificity is only moderate. This shows that there may be a relevant proportion of false positive results and that positive test results from PE analysis need to be confirmed by more specific tests.

Discussion

This study could show that PD-L1 analysis from PE samples of NSCLC patients is an attractive diagnostic tool with a very high sensitivity and negative predictive value of up to 100%.

In clinical routine immunocytochemistry based PD-L1 analysis from malignant PE may be of great relevance. Particularly in patients with an increased risk of morbidity and mortality from invasive procedures such as thoracoscopy or bronchoscopy, the less invasive procedure of pleural puncture may allow a decision-making process for tumor specific therapy.

With PD-L1 expression levels of TPS ≥50% defined as PD-L1-positive, it could be demonstrated that results from PE showed a high sensitivity (100%), negative predictive value (100%) and negative likelihood ratio (0.00) with an acceptable specificity (63%) and a limited positive predictive value (45%). Given that PE was tumor cell positive and PD-L1 analysis was feasible, no false negative results were documented. These statistical data suggest that negative results are highly reliable. The EMA approval for Pembrolizumab as a single agent 1st-line therapy regimen was granted for patients with a TPS ≥50%. Reflecting these considerations, it can be concluded, that with negative PD-L1 analysis (TPS <50%) from PE samples a checkpoint inhibitor monotherapy is not feasible. The more invasive thoracoscopy will not add new information.

Considering these data, a clinical algorithm may be developed which is shown in figure 2.

The same conclusions have to be drawn regarding the clinical indication and FDA approval of Atezolizumab as a single agent 1st-line therapy.

However, tissue sampling by thoracoscopy or bronchoscopy should be considered when cytological analysis from PE does not detect tumor cells, when results remain inconclusive or when PD-L1-analysis is not feasible. This sequential approach is supported by the data from this study. Although results from PE samples were negative or inconclusive, PD-L1 positive tumor cells could be detected by thoracoscopy and/or bronchoscopy in some patients.

When PD-L1 expression levels with a TPS ≥1% were defined as PD-L1-positive, the sensitivity was good but lower (86%) with an associated moderate positive predictive value (75%), limited negative predictive value (60%) and low specificity (43%). The FDA approval was granted for Pembrolizumab as a single agent 1st-line therapy with a TPS ≥1%.

Under these conditions, it should be considered to dispense with PD-L1 analysis in PE samples within the FDA-regulated region. Other invasive procedures should be favored instead. Thoracoscopy or bronchoscopy may be more suitable to clarify the clinical indication for a single agent 1st-line immune checkpoint inhibitor therapy.

Overall, the data underlines the importance of applying appropriate diagnostic approaches in accordance with sensitivity and specificity values.

In some patients within this study, malignant cells were detected in PE and showed PD-L1 positivity, while the corresponding pleural tissue samples were free of malignancy. In clinical practice, such a constellation may prompt tumor tissue

sampling from other sites in order to further substantiate the results from PD-L1 analysis.

To our knowledge this represents the first prospective study where sensitivity of PD-L1 analysis in PE has been determined. Overall, there is only limited evidence on the diagnostic significance of PD-L1 analysis in PE samples.

Grosu et al. analyzed a cohort of 82 subjects and reported a good correlation and concordance of PD-L1 results from PE and histological specimens with kappa values of 0.76 and 0.78 [8]. However, due to the retrospective study design the data were heterogenous as the samples were obtained under different clinical conditions. Some of the histological samples represented core biopsies from the primary tumor site, others represented material from surgical resections whereas cytological specimens where obtained from PE. The time between histological and cytological sampling ranged from 0 to 363 days. At least one chemotherapy treatment was applied between surgical specimen collection and PE collection in 45% of patients. Due to the retrospective design, it cannot be ruled out that the results may be influenced by the different biological conditions of the neoplasm at the timepoint and location of tissue and PE sampling, respectively. In another retrospective study, 29 paired PE and tissue samples were analyzed [9]. A history of chemotherapy was documented in 30% of the cases. With the limited number of samples and the given heterogeneity of the data conclusions have to be drawn with caution, however the authors reported concordance between PE and histology results in 25/29 cases. Zou et al. retrospectively analyzed the concordance of PD-L1 results in PE and histology samples obtained from various sites in 124 subjects [10]. Although the PD-L1 expression was concordant in 86% of cases, a significant difference of expression levels was determined. This difference was reduced by a not yet validated TTF1 plus PD-L1 double staining protocol.

Considering that two novel approaches have been performed in this study, the results need to be further substantiated. Another retrospective study analyzed 51 cases by comparing PD-L1 results from PE and pleural biopsies using a novel PD-L1 scoring system [11]. The overall correlation was substantial, however the differences were significant for high PD-L1 expression levels.

An accurate interpretation of PD-L1 immunocytochemistry remains essential. Particularly in the event of a low proportion of PD-L1-positive cells in immunocytochemistry analysis, it should be noted that false-positive results may be obtained, as staining of mesothelial cells may also occur, which cannot always be clearly distinguished from tumor cells outside a tissue context [12-14]. This may partially explain the limited specificity of PD-L1 analysis, especially when a TPS ≥1% is regarded as positive.

While the results of this study suggest that PD-L1 analysis from PE samples is not suited to completely replace immunohistochemistry analysis of corresponding pleura tissue samples, it can be of additive value. The fact that the PE did not always yield enough material for a comprehensive immunocytochemistry evaluation including PD-L1 analysis, constitutes an important limitation, in part leading to inconclusive results regarding malignancy. This may be due to a low rate of cells scaling off from the pleural lesion and/or a low rate of migration of tumor cells into the pleural fluid.

As sensitivity and specificity analyses, in their nature, rely on the definition of a reference standard, we chose the immunohistochemistry analysis of pleural tissue for this purpose. In theory, as opposed to cytological preparations of PE, thoracoscopically obtained pleural tissue samples from suspected tumor sites should offer a higher yield of good quality sample material. Based on those, it should be

possible to successfully characterize the specific features of each individual lung carcinoma, including PD-L1 expression. Tumor cells within the malignant PE can be assumed to have scaled off from pleural lesions and thus their characteristics should closely resemble those of the histological samples. Thus, evaluating the results from immunocytochemistry analysis of pleural punctate compared to pleural tissue samples as the reference standard seems reasonable. On the other hand, a certain degree of tumor heterogeneity should be considered, which might cause differences in PD-L1 expression [15].

Conclusion

PD-L1 analysis in tumor positive PE samples is characterized by a very high sensitivity and negative predictive value, especially regarding PD-L1 expression with a TPS ≥50%. Negative results based on this cut-off seem very reliable and could thus help in the decision against a first-line checkpoint inhibitor monotherapy in the EMA region. Within the FDA-regulated region, however, this analysis might be less reliable, as the results are judged mainly based on a 1% cut-off level, which leads to a markedly lower negative predictive value. This indicates the necessity to perform corresponding immunohistochemistry analysis of pleural tissue, bearing in mind the costs for the associated therapy.

Further studies are necessary to enhance the confidence of our results, to further assess the role of PD-L1 analysis based on additional bronchoscopy and to investigate the potential issue of false-positive immunocytochemistry results.

Figure 1

Flow chart depicting numbers of different samples and corresponding diagnostic procedures

Legend:

PD-L1: programmed cell death protein ligand 1

Figure 2

Proposed clinical algorithm for PD-L1 testing in NSCLC patients with pleural effusion

Legend:

NSCLC	Non-small cell lung cancer
PD-L1	programmed cell death protein ligand 1

Table 1

Anthropometric data and smoking status of 50 patients included

Gender (number [percent]) female male	 17 [34%] 33 [66%]
Age (Years – median [quartile1; quartile3])	72.5 [62.8; 76.3]
Body mass index (kg/m² – median [quartile1; quartile3])	25.7 [23.2; 28.5]
Smoking status (number [percent]) never ex-smoker current smoker	 7 [14%] 28 [56%] 15 [30%]

Table 2

Summary of malignancy and PD-L1 status

	Pleural effusion (n)	Pleural biopsy (n)
Number of cases	50	50
Malignancy (+/-/○)	36/ 7/ 7	40/ 10/ 0
PD-L1 ≥1% of TC (+/-/nd)	19/ 6/ 25	21/ 14/ 15
PD-L1 ≥50% of TC (+/-/nd)	13/ 12/ 25	8/ 27/ 15

Legend:

+ = positive | - = negative | ○ = inconclusive | nd = not done | TC = tumor cells

Table 3

PD-L1 detection in pleural effusion based on all cases with successful PD-L1 analysis:

a) Cross-classification of pleura biopsy and pleura effusion analysis results concerning PD-L1 detection;

b) Sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio, negative likelihood ratio

PD-L1 expression ≥50% of tumor cells defined as positive				
a)				
		Pleural biopsy		∑
		positive	negative	
Pleural effusion	positive	5	6	11
	negative	0	10	10
∑		5	16	21
b)				
Parameter		Estimate	95% Confidence interval	
Sensitivity		100%	46-100%	
Specificity		63%	36-84%	
Positive Predictive Value		45%	18-75%	
Negative Predictive Value		100%	66-100%	
Positive Likelihood Ratio		2.67	1.42-5.02	
Negative Likelihood Ratio		0.00	Not defined	

PD-L1 expression ≥1% of tumor cells defined as positive				
a)				
		Pleural biopsy		∑
		positive	negative	
Pleural effusion	positive	12	4	16
	negative	2	3	5
∑		14	7	21
b)				
Parameter		Estimate	95% Confidence interval	
Sensitivity		86%	56-97%	
Specificity		43%	12-80%	
Positive Predictive Value		75%	47-92%	
Negative Predictive Value		60%	17-93%	
Positive Likelihood Ratio		1.50	0.76-2.95	
Negative Likelihood Ratio		0.33	0.07-1.56	

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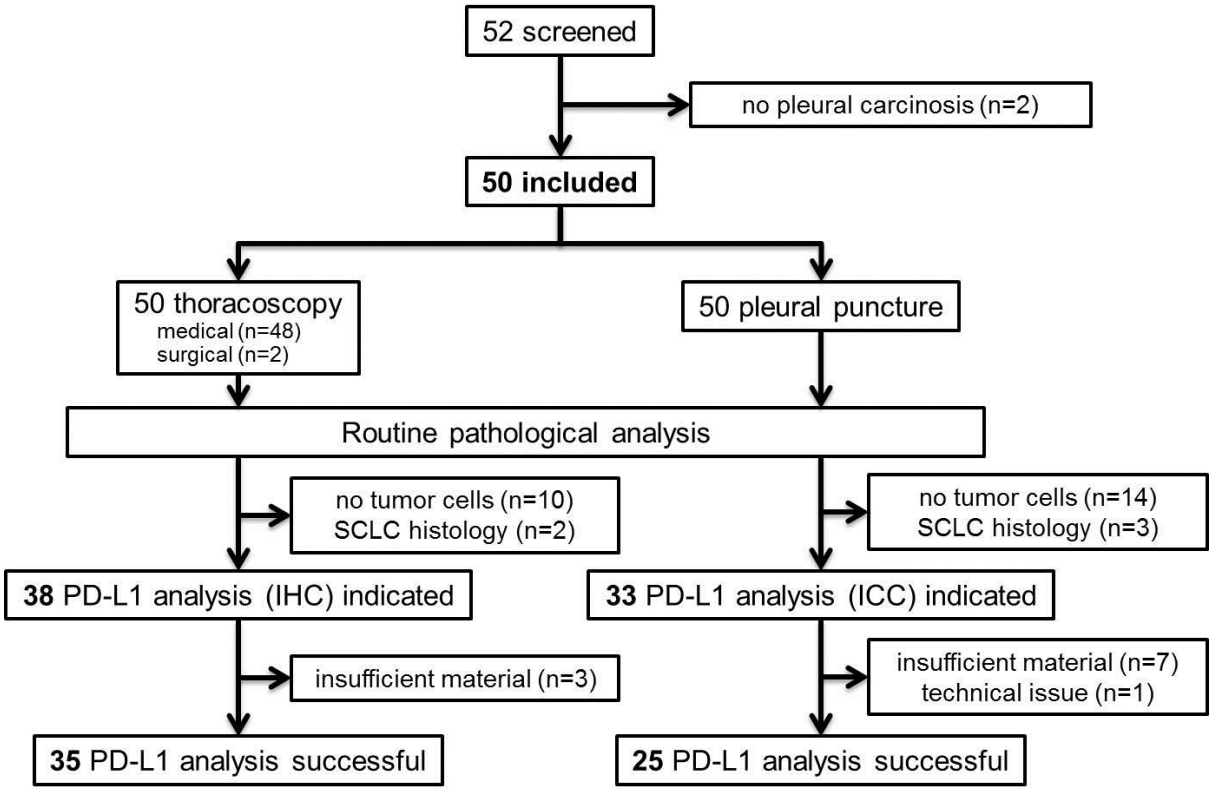
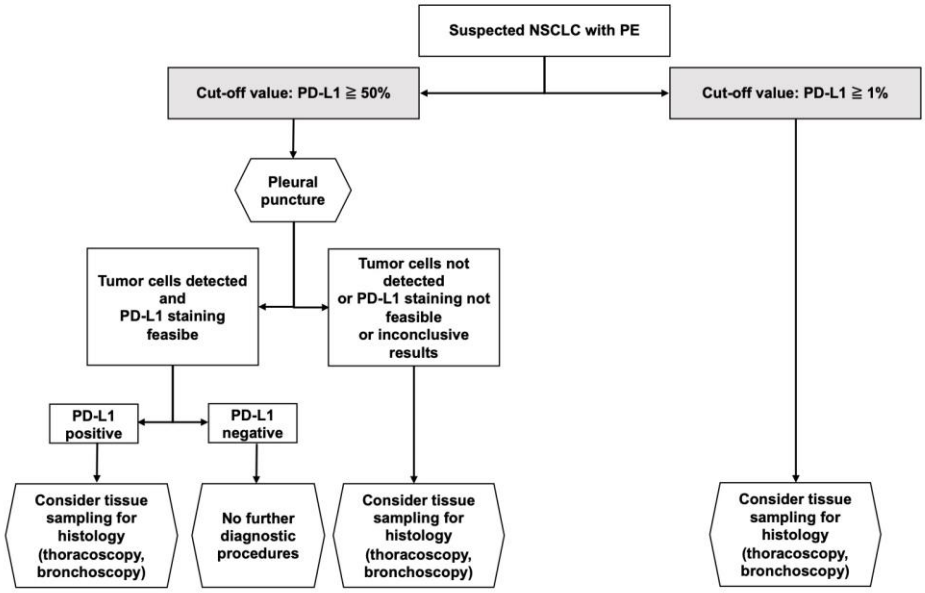

52 screened
no pleural carcinosis (n=2)
50 included
50 thoracoscopy
medical (n=48)
surgical (n=2)
50 pleural puncture
Routine pathological analysis
no tumor cells (n=10)
SCLC histology (n=2)
no tumor cells (n=14)
SCLC histology (n=3)
38 PD-L1 analysis (IHC) indicated
33 PD-L1 analysis (ICC) indicated
insufficient material (n=3)
insufficient material (n=7)
technical issue (n=1)
35 PD-L1 analysis successful
25 PD-L1 analysis successful


Suspected NSCLC with PE
Cut-off value: PD-L1 ≧ 50%
Cut-off value: PD-L1 ≧ 1%
Pleural puncture
Tumor cells detected and PD-L1 staining feasibe
Tumor cells not detected or PD-L1 staining not feasible or inconclusive results
PD-L1 positive
PD-L1 negative
Consider tissue sampling for histology (thoracoscopy, bronchoscopy)
No further diagnostic procedures
Consider tissue sampling for histology (thoracoscopy, bronchoscopy)
Consider tissue sampling for histology (thoracoscopy, bronchoscopy)

Appendix

Table 1

Medical treatment in metastatic NSCLC (UICC IV) targeting the PD1/PD-L1-pathway, approvals according to European Medicines Agency (EMA) and United States Food and Drug Administration (FDA).

Medical Treatment (target)	**EMA approval**	**FDA approval**
Pembrolizumab (PD-1)	as a single agent for the 1st-line treatment in metastatic NSCLC expressing PD-L1 [Tumor Proportion Score (TPS) ≥50%] with no EGFR or ALK genomic tumor aberrations	as a single agent for the 1st-line treatment in NSCLC expressing PD-L1 [Tumor Proportion Score (TPS) ≥1%] as determined by an FDA-approved test, with no EGFR or ALK genomic tumor aberrations, and is: stage III where patients are not candidates for surgical resection or definitive chemoradiation, or metastatic.
	in combination with Pemetrexed and platinum chemotherapy, as 1st-line treatment in metastatic nonsquamous NSCLC, with no EGFR or ALK genomic tumor aberrations.	
	in combination with carboplatin and either paclitaxel or paclitaxel protein-bound, as 1st-line treatment in metastatic squamous NSCLC.	
	as a single agent for treatment in metastatic NSCLC expressing PD-L1 (TPS ≥1%) after at least one prior chemotherapy regimen. Patients with EGFR or ALK genomic tumor aberrations should have received targeted therapy prior to receiving Pembrolizumab.	as a single agent for treatment in metastatic NSCLC expressing PD-L1 (TPS ≥1%) as determined by an FDA-approved test, with disease progression on or after platinum-containing chemotherapy. Patients with EGFR or ALK genomic tumor aberrations should have disease progression on FDA-approved therapy for these aberrations prior to receiving Pembrolizumab.
Nivolumab (PD-1)	-	In combination with Ipilimumab and two cycles of platinum-doublet chemotherapy for 1st-line treatment in metastatic or recurrent NSCLC with no EGFR or ALK genomic tumor aberrations.
	-	In combination with Ipilimumab for 1st-line treatment in metastatic NSCLC expressing PD-L1 (TPS ≥1%) as determined by an FDA-approved test, with no EGFR or ALK genomic tumor aberrations.
	As single agent for treatment of locally advanced or metastatic	As a single agent for treatment in metastatic NSCLC with disease

	NSCLC after prior chemotherapy	progression on or after platinum-containing chemotherapy. Patients with EGFR or ALK genomic tumor aberrations should have disease progression on FDA-approved therapy for these aberrations prior to receiving Nivolumab.
Atezolizumab (PD-L1)	-	as a single agent for 1st-line treatment in metastatic NSCLC expressing PD-L1 (PD-L1 ≥ 50% of tumor cells [TC ≥ 50%] or PD-L1 stained tumor-infiltrating immune cells [IC] covering ≥ 10% of the tumor area [IC ≥ 10%]), as determined by an FDA-approved test, with no EGFR or ALK genomic tumor aberrations.
	in combination with bevacizumab, paclitaxel and carboplatin for the 1st-line treatment in metastatic non-squamous NSCLC with no EGFR or ALK genomic tumor aberrations.	
	in combination with paclitaxel protein-bound and carboplatin for 1st-line treatment in metastatic non-squamous NSCLC with no EGFR or ALK genomic tumor aberrations.	
	as a single agent for treatment in locally advanced or metastatic NSCLC after prior chemotherapy. Patients with EGFR or ALK genomic tumor aberrations should have received targeted therapy prior to receiving atezolizumab.	as a single agent for treatment in metastatic NSCLC with disease progression on or after platinum-containing chemotherapy. Patients with EGFR or ALK genomic tumor aberrations should have disease progression on FDA-approved therapy for these aberrations prior to receiving atezolizumab.

Legend			
ALK	Anaplastic lymphoma kinase	NSCLC	Non-small cell lung cancer
EGFR	Epidermal growth factor receptor	PD-1	Programmed cell death protein 1
EMA	European Medicines Agency	PD-L1	Programmed cell death 1 ligand 1
FDA	United States Food and Drug Administration	TPS	Tumor Proportion Score
		TC	Tumor cells
IC	Immune cells	UICC	Union for international cancer control

Appendix

Table 2. Per-patient overview of malignancy and PD-L1 status in different samples

Subject no.	Pleural effusion			Pleural tissue (thoracoscopy)			Bronchoscopic samples			Histology
	Detection of malignancy	PD-L1 ≥1% of TC	PD-L1 ≥50% of TC	Detection of malignancy	PD-L1 ≥1% of TC	PD-L1 ≥50% of TC	Detection of malignancy	PD-L1 ≥1% of TC	PD-L1 ≥50% of TC	
1	○	□		−	□		+	□		Squamous
2	+	+	+	−	□		□			Adeno
3	○	□		+	+	−	+	□		Adeno
4	+	+	−	+	+	−	□			Adeno
5	−	□		−	□		+	□		NHL
6	+	−	−	+	□		+	−	−	Adeno+SCLC
7	+	+	+	+	+	−	+	□		Adeno
8	+	−	−	+	−	−	□			Adeno
9	○	□		+	+	+	□			SM
10	+	□		+	□		+	□		SCLC
11	+	+	+	+	+	+	+	−	−	Adeno
12	○	□		−	□		+	+	+	Adeno
13	+	□		+	−	−	+	□		Adeno
14	+	+	−	−	□		+	+	−	Ambiguous
15	+	+	+	+	+	−	+	+	−	Adeno
16	+	□		+	□		□			Adeno
17	+	+	−	+	−	−	□			Adeno
18	+	+	−	+	+	−	+	+	+	Adeno
19*	−	□		−	□		□			Adeno
20	+	+	+	+	+	−	+	□		Adeno
21	+	□		+	□		□			SCLC
22	○	□		+	−	−	+	□		Squamous
23	−	□		+	+	−	+	□		Squamous
24	+	□		−	□		+	□		SCLC
25	+	+	+	+	+	+	+	□		Adeno
26	+	+	−	+	−	−	□			Adeno
27	−	□		+	−	−	□			LCNEC
28	○	□		−	□		+	□		Adeno
29	−	□		+	+	−	−	□		Squamous
30	+	+	+	+	+	+	+	□		Adeno
31	+	−	−	+	+	−	+	□		Adeno
32	+	+	−	+	+	−	+	□		Adeno
33	+	□		+	+	+	+	□		Adeno
34	○	□		+	−	−	+	□		Squamous
35	−	□		−	□		+	+	−	Squamous
36	−	□		−	□		+	□		SCLC
37	+	−	−	+	−	−	+	□		Ambiguous
38	+	□		+	+	+	+	□		Adeno
39	+	+	+	+	+	+	−	□		Adeno
40	+	+	+	+	−	−	−	□		Adeno
41	+	−	−	+	−	−	+	□		Squamous
42	+	+	+	+	□		+	+	−	Adeno
43	+	□		+	+	−	+	+	−	Adeno
44	+	+	+	+	+	−	+	□		Adeno
45	+	−	−	+	+	−	+	+	−	Adeno
46	+	+	+	+	−	−	+	□		Adeno
47	+	□		+	−	−	+	□		Adeno
48	+	+	+	+	+	+	+	+	+	Adeno
49	+	□		+	−	−	+	−	−	Adeno
50	+	□		+	−	−	+	□		Adeno
□ not done	0	25	0	0	15	0	10	28	0	
+ positive	36	19	13	40	21	8	37	9	3	
− negative	7	6	12	10	14	27	3	3	9	
○ inconclusive	7	0	0	0	0	0	0	0	0	

TC = tumor cells | NHL = non-Hodgkin lymphoma | SM = sarcomatoid mesothelioma | SCLC = small-cell lung carcinoma
* This patient in whom all samples available in the current analysis were free of malignancy presented with a pre-existing diagnosis of adenocarcinoma.

Appendix

Table 3

PD-L1 detection in pleural effusion based on all cases with indication for PD-L1 analysis

a) Cross-classification of pleura biopsy and pleura effusion analysis results concerning PD-L1 detection;

b) Sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio, negative likelihood ratio

PD-L1 expression ≥50% of tumor cells defined as positive				
a)				
		Pleural biopsy		∑
		positive	negative	
Pleural effusion	positive	5	7	12
	negative	2	17	19
∑		7	24	31

b)			
Parameter	Estimate	95% Confidence interval
Sensitivity	71%	30-95%
Specificity	71%	49-87%
Positive Predictive Value	42%	16-71%
Negative Predictive Value	89%	65-98%
Positive Likelihood Ratio	2.45	1.12-5.34
Negative Likelihood Ratio	0.40	0.12-1.35

PD-L1 expression ≥1% of tumor cells defined as positive				
a)				
		Pleural biopsy		∑
		positive	negative	
Pleural effusion	positive	12	5	27
	negative	5	9	14
∑		17	14	31

b)			
Parameter	Estimate	95% Confidence interval
Sensitivity	71%	44-89%
Specificity	64%	36-86%
Positive Predictive Value	71%	44-89%
Negative Predictive Value	64%	36-86%
Positive Likelihood Ratio	1.98	0.92-4.26
Negative Likelihood Ratio	0.46	0.20-1.05