




ORIGINAL ARTICLE

Choice of fixative affects programmed death-ligand 1 expression in cell blocks from pleural effusions with metastatic pulmonary adenocarcinoma

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Abstract

Background: Programmed death-ligand 1 (PD-L1) immunocytochemical (ICC) analysis of cell blocks (CBs) has recently emerged in clinical practice. Unlike standardized immunohistochemistry on formalin-fixed, paraffin-embedded tissues, cytology involves various preparation methods and fixatives. This study investigated how various fixatives influence PD-L1 immunoreactivity in CBs from malignant pleural effusions (MPEs) with metastatic pulmonary adenocarcinoma (AC).

Methods: Thirty-three MPEs from patients with pulmonary AC were prospectively included. Four matched CBs per case were fixed in four different fixatives and immunostained with three PD-L1 antibodies. Tumor proportion score and staining intensity were evaluated at multiple cutoffs.

Results: The cytology–cytology correlation of PD-L1 expression with the antibodies 28-8, 22C3, and SP263 was assessed in matched CBs fixed in either formalin,

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PreservCyt, CytoLyt, or CytoRich Red (the latter only in 26 cases). Compared to formalin, PreservCyt and CytoLyt showed moderate concordance at the $\geq 1\%$ cutoff (Cohen kappa [κ], 0.463–0.535 and 0.57–0.586, respectively), except SP263 with CytoLyt, which demonstrated only fair concordance (κ , 0.382). The corresponding figures for CytoRich Red indicated substantial concordance for 28-8 and SP263 (κ , 0.601 and 0.669) and very good concordance for 22C3 (κ , 0.806). At the $\geq 50\%$ cutoff, concordance improved for 28-8 and 22C3 but remained largely unchanged for SP263. All alcohol-based fixatives produced significantly weaker PD-L1 staining intensity than formalin across all antibodies ($p < .001$ –.007).

Conclusions: PD-L1 ICC expression in CBs depends on the fixative and antibody used. Alcohol-based fixatives, particularly with low cutoffs, risk underestimating PD-L1 positivity, and may contribute to false-negative results. ICC protocol optimization is essential before diagnostic use.

KEYWORDS

cell block, cytology–cytology correlation, CytoLyt, CytoRich Red, formalin, PreservCyt, programmed death-ligand 1 (PD-L1)

INTRODUCTION

In clinical trials, programmed death-ligand 1 (PD-L1) assessment has generally been accepted on formalin-fixed, paraffin-embedded (FFPE) tissues,^{1–9} which has limited validation in treatment studies to biopsies and resected samples. However, many lung cancer cases are diagnosed solely via cytology,^{10,11} including malignant pleural effusions (MPEs), with metastatic pulmonary adenocarcinoma (AC) being the most common and often serving as the first available diagnostic material for pulmonary AC.^{12,13} Also, cytology is often the only available diagnostic material, and provides sufficient samples for ancillary techniques.^{13–15} As a result, immunohistochemical (IHC) evaluation of PD-L1 in cytology has gained attention since the introduction of immunotherapy,^{16–18} with studies analyzing the reliability of cytology compared to histology.^{19–23} Subsequently, PD-L1 testing in cytology has become an essential part of laboratory practice, with growing interest in using cytological cell blocks (CBs) for evaluation. A 2019 global survey by the International Association for the Study of Lung Cancer Pathology Committee found that 75% of participants used CBs and 11% used smears for PD-L1 testing.²⁴

Whereas IHC analysis on FFPE tissues follows standardized protocols, with formaldehyde as a fixative and considered the gold standard, cytology uses diverse techniques with varying fixation and preparation methods, which makes the procedure more challenging. In 2020, a European Federation of Cytology Societies survey found that 38% of European laboratories used CBs for immunocytochemical (ICC) analysis, either alone or with other preparations, and 96% of laboratories performed ICC assays with protocols identical to those for FFPE samples.²⁵ Furthermore, a recent survey found that low cellularity and dispersed cells are the most common CB issues in daily cytology, regardless of preparation method or sample selection.²⁶ In addition to multiple CB preparation methods (e.g., plasma-

thrombin, agar, Cellient, and Shandon),^{26–28} there are different fixatives, including commercially available liquid-based cytology (LBC) fixatives (e.g., CytoLyt, PreservCyt, and CytoRich Red). Nevertheless, CBs enhance morphological detail and are widely used for biomarker analysis, particularly ICC analysis.^{15,27,28}

Recently, two new guidelines from the College of American Pathologists (CAP) provided details on PD-L1 testing in cytology. A joint effort of the CAP, the International Association for the Study of Lung Cancer, the American Society of Clinical Oncology, the Association for Molecular Pathology, the Pulmonary Pathology Society, and the patient advocacy LUNgevity Foundation led to guidelines on PD-L1 and tumor mutation burden testing in lung cancer, with emphasis on the need for cytology validation for PD-L1, particularly when using alcohol-based fixatives.²⁹ Additionally, recent CAP guidelines³⁰ updated the 2014 recommendations³¹ on IHC assay validation. The importance of validating PD-L1 testing for various cytological methods, including alcohol-fixed, paraffin-embedded CBs, is highlighted. Any cytological preparation differing from FFPE handling should undergo validation before clinical use.³⁰ Despite the effect of alcohol on PD-L1 staining having been discussed in several cytological–histological PD-L1 correlation studies on lung cancer,^{19–23} findings remain controversial and inconclusive. Each laboratory can address this by verification of its protocols, and this study aims to shed light on the process. Therefore, this prospective study aimed to examine the effects of four commonly used fixatives (formalin, PreservCyt, CytoLyt, and CytoRich Red) on ICC staining with three PD-L1 antibodies (28-8, 22C3, and SP263) in matched pleural effusion CBs from patients with metastatic pulmonary AC by assessing staining proportion and intensity across quadruple-matched CBs. The same approach was used in a previous study evaluating a different set of diagnostic biomarkers.³² The present study expands on that cohort by focusing on PD-L1 and introducing an incrementally novel aspect by

assessing not only diagnostic markers but also clinically relevant cut-offs for the predictive biomarker PD-L1.

MATERIALS AND METHODS

Study design and specimen selection

This prospective study was a follow-up with an overlapping cohort, with an expansion of the previous study population from our institution. It includes consecutive MPEs from patients with metastatic pulmonary AC, collected prospectively at the Department of Pathology and Cytology, Halland Hospital Halmstad, Sweden. The first 26 cases were collected between December 2020 and April 2023, of which 24 were reported previously.³² The present study includes these cases plus seven additional consecutive cases collected from May 2023 to September 2024. MPEs that showed only a few tumor cells in May-Grünwald-Giemsa-stained smears or in hematoxylin-eosin (H & E)-stained CB sections, or those with cell pellets that were too small to be divided into four separate CBs, were excluded. Additionally, specimens with fewer than 100 malignant cells on the H & E slide, as well as those with diagnoses other than pulmonary AC, were also excluded. Four matched CBs from each pleural effusion were prepared and fixed with four distinct fixatives: 10% neutral buffered formalin, considered the gold standard; PreservCyt (Hologic Inc, Marlborough, Massachusetts); CytoLyt (Hologic Inc); and CytoRich Red (Becton, Dickinson and Company, Franklin Lakes, New Jersey). The initial seven cases were fixed with only the first three fixatives listed above (the first cases were not fixed with CytoRich Red because this fixative was not included in the initial part of the study; however, it was incorporated later). There were no differences in the sampling, preparation, or fixation methods among the cases. A schematic illustration of the CB preparation procedure, with the simple sedimentation technique and in part the Shandon Cytoblock method (Thermo Scientific, Cheshire, UK) according to the manufacturer's instructions,³³ is shown in Figure 1. The approach is also described in a previous study.³² For further details on preparation and diagnostics, see Supporting Information.

Ethics statement

The study adhered to the Declaration of Helsinki, and received approval from the Regional Ethical Review Board of the Southern Health Care Region in Lund (protocol codes 2024-06821-01 and 2006-399, with additional approval 2017-708). Informed consent was obtained from all subjects involved in the study.

Immunostaining of specimens

Sections from all matched CB preparations were stained with the same PD-L1 antibodies for each case. Immunostaining for PD-L1 28-8 and

22C3 was performed at the Department of Pathology and Cytology in Halmstad, whereas immunostaining for PD-L1 SP263 was conducted at the Department of Pathology and Cytology, Kalmar County Hospital, Kalmar, Sweden. The staining procedures followed the same protocols used in the clinical diagnostic setting at the pathology departments. Details on the antibodies used, including the automation system, incubation time, and dilution, are provided in Table 1. Further details on the ICC staining procedures can be found in Supporting Information.

Evaluation of immunostainings

All slides were evaluated with a conventional light microscope. Cases with more than 100 viable tumor cells were deemed adequate, and tumors were classified as positive on the basis of the reactivity of viable malignant cells on the entire slide, regardless of staining intensity. For all PD-L1 antibodies, only membranous staining was regarded, whereas any cytoplasmic or granular staining was disregarded. Reactivity observed in nonmalignant cells, necrotic areas, immune cells, or macrophages was also disregarded.

The ICC stains were categorized as negative (score 0, <1%) or positive on the basis of six tumor proportion score (TPS) levels: score 1, $\geq 1\%$ –4%; score 2, $\geq 5\%$ –9%; score 3, $\geq 10\%$ –24%; score 4, $\geq 25\%$ –49%; score 5, $\geq 50\%$ –74%; and score 6, $\geq 75\%$ –100%. Staining intensity was evaluated separately and scored as follows: 0 for no reactivity, + for weak staining, ++ for moderate staining, and +++ for strong staining, with the predominant intensity of immunoreactivity across the entire slide being considered.

The assessment of immunostains was facilitated by correlation with H & E staining and other available immunostains from routine workup or newly stained slides of each CB with epithelial cell adhesion molecule (clone BerEp4; dilution, ready to use; Dako/Agilent, Glostrup, Denmark) and/or thyroid transcription factor 1 (clone SPT24; dilution, 1:50; Leica Biosystems/Newcastle, Ltd, Newcastle upon Tyne, UK). The scoring was conducted independently and blindly by five separate examiners, with no side-by-side comparisons. Initially, a certified cytotechnologist (M.S.I.M.) assessed the slides, followed by four experienced cytopathologists (L.P., T.S., L.M.A., and I.K.). Additionally, one other experienced cytopathologist (R.P.) scored a large number of cases. The most agreed-upon score among the examiners for each staining reactivity was selected. Possible reasons for discrepancies between examiners or between CB preparations from the same specimen were discussed, and consensus scores were applied.

Statistical analysis

The frequency and intensity of positivity were compared via Friedman tests to identify any systematic differences among all examined fixatives simultaneously. This was followed by McNemar tests for pairwise comparisons of fixatives at a single cutoff, whereas the Wilcoxon signed-rank test was used for multiple cutoff levels or scores by analyzing each antibody individually. McNemar and Wilcoxon signed-

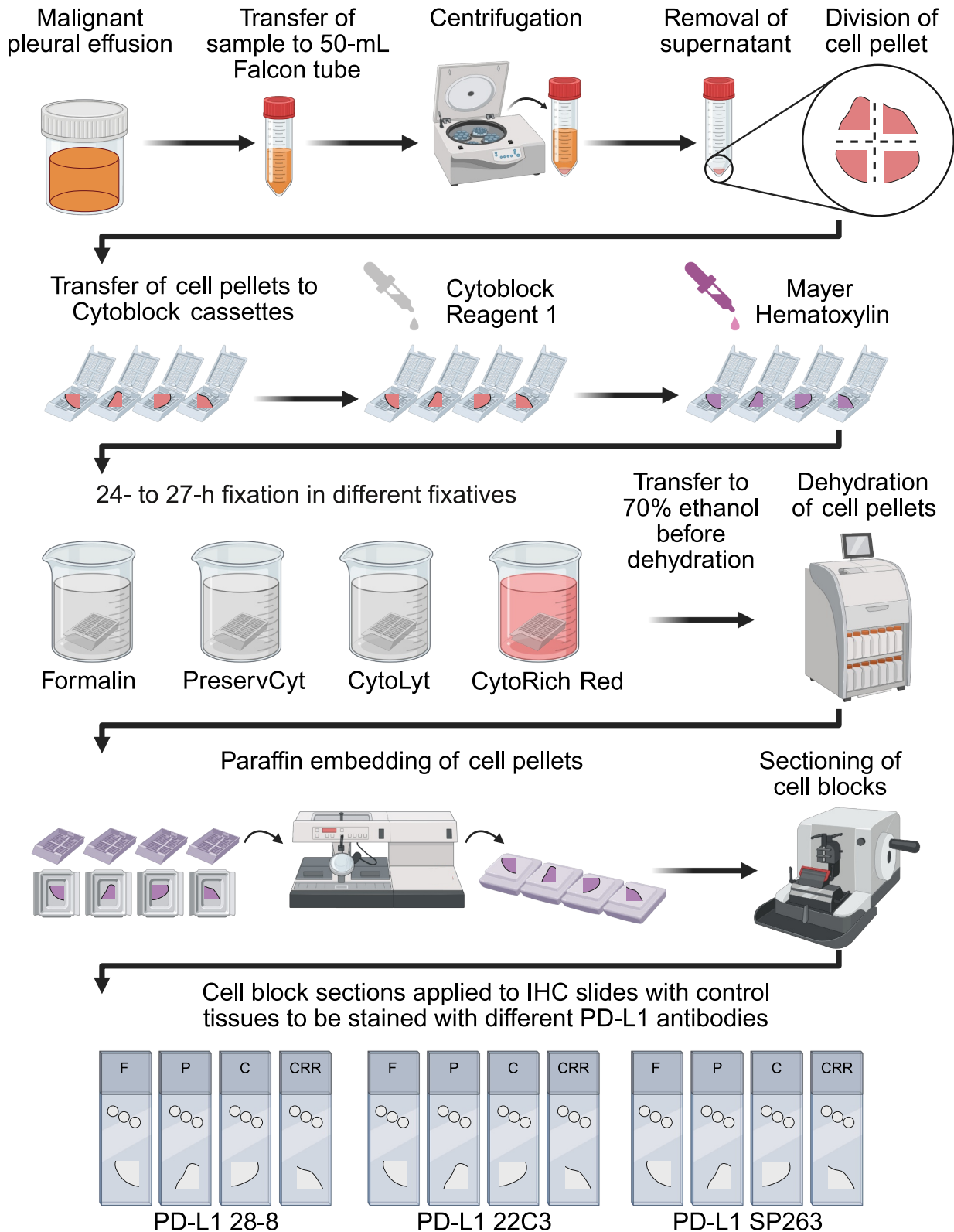


FIGURE 1 Schematic overview of the preparation procedure of the cell blocks used in this study. This illustration depicts the cell block preparation method of pleural effusion with four different fixation media (formalin and three liquid-based cytology fixatives) for 24–27 h. C indicates CytoLyt; CRR, CytoRich Red; F, formalin; IHC, immunohistochemistry; P, PreservCyt; PD-L1, programmed death-ligand 1. Created with BioRender.com.

TABLE 1 Detailed characteristics and description of the immunocytochemical stainings used.

Biomarker (antibody)	Vendor	Dilution	Pretreatment	Visualization system	Staining platform	Control tissue	
						Positive control	Negative control
PD-L1 (28-8)	Dako/Agilent Technologies, Glostrup, Denmark	RTU	TRS, low pH, 20 min, 97°C	EnVision FLEX visualization system	Autostainer Link 48, Dako, Agilent	Tonsil (+ in histiocytes of germinal centers; ++ in crypt epithelium), placenta (+++), and cell line (NCI-H226) ^a	Appendix, cell line (MCF-7), ^a and NCR control ^b
PD-L1 (22C3)							
PD-L1 (SP263)	Ventana Medical Systems Inc, Tucson, Arizona		CC1, 64 min, 100°C	OptiView visualization system	BenchMark ULTRA, Ventana, Roche		

Note: Scoring scale (dominating intensity): +, weak expression; ++, moderate expression; +++, strong expression.

Abbreviations: CC1, cell conditioning 1; NCR, negative control reagent; PD-L1, programmed death-ligand 1; RTU, ready to use; TRS, target retrieval solution.

^aA control slide produced from two cell lines, NCI-H226 (PD-L1 positive) and MCF-7 (PD-L1 negative), included in the antibody kit, was used for each run of PD-L1 antibodies 28-8 and 22C3.

^bAn additional negative antibody control for every sample slide from an identical slide of each specimen was stained with NCR, a buffer containing immunoglobulin G antibodies that lack specificity for PD-L1 and serving as an isotype control. It was used for almost all cases stained with PD-L1 antibodies 28-8 and 22C3.

rank tests were applied only to significant differences identified by the Friedman test with p values of $<.05$. Also, overall percentage agreement (OPA), positive percentage agreement (PPA), and negative percentage agreement (NPA) were calculated for pairwise comparisons of the fixatives, with formalin fixation as the nonreference standard, and with positive staining defined as $\geq 1\%$, $\geq 5\%$, $\geq 10\%$, $\geq 25\%$, $\geq 50\%$, or $\geq 75\%$ positive tumor cells. In addition, the diagnostic concordance of expression was evaluated with unweighted Cohen kappa (κ) agreement statistics (with bootstrapped 95% confidence intervals [CIs]) for pairwise fixative comparisons (2×2 tables) and weighted kappa ($W\kappa$) for all matched CB preparations/fixatives (4×4 or 7×7 tables). Following the terminology of Altman³⁴ and Landis and Koch,³⁵ the κ and $W\kappa$ values were classified as poor/slight agreement (≤ 0.20), fair agreement (0.21–0.40), moderate agreement (0.41–0.60), good/substantial agreement (0.61–0.80), or very good/almost perfect agreement (0.81–1.00).

All p values were calculated via two-sided tests, with p values of $>.05$ considered not statistically significant. No correction for multiple comparisons was applied because the analyses were primarily exploratory in nature. CIs were determined via a modified Wald method with a 95% Wilson score, calculated according to the website <https://www.graphpad.com/quickcalcs/> (accessed July 7, 2025). Descriptive analyses, data evaluations, and summary graphs were generated with SPSS Statistics for Windows, version 30.0 (IBM, Armonk, New York).

RESULTS

Characteristics of the specimens

A total of 109 consecutive MPEs were collected during the study period from 82 different patients. On the basis of the inclusion

criteria, 35 patients were excluded because of a final diagnosis of a malignancy other than pulmonary AC. Among the remaining 47 cases, 33 had sufficient material in all CBs and were included in the study. Three CBs, fixed in formalin, PreservCyt, and CytoLyt, were prepared for each of the first seven cases. Four CBs, including one with an additional fixation in CytoRich Red, were prepared for each of the remaining 26 cases. A flowchart illustrating the specimen collection process is shown in Figure 2.

All matched CBs meeting the study criteria were analyzed for ICC expression of three PD-L1 antibodies (28-8, 22C3, and SP263). Among the analyzed cases, nine (27%) were male, 24 (73%) were female, and the median age at diagnosis was 73 years (range, 43–91 years). Twenty-three (70%) of the included patients received oncological therapy. Among them, 18 specimens (78%) were obtained before any treatment, two (9%) were collected during treatment, and three (13%) were obtained after treatment.

Frequency of PD-L1 positivity in matched CBs

PD-L1 expression with the antibodies 28-8, 22C3, and SP263 was evaluated in matched CBs from 33 MPEs, fixed in either formalin, PreservCyt, CytoLyt, or CytoRich Red (the latter in 26 cases only). Table 2 summarizes PD-L1 immunoreactivity at six different cutoffs for positive tumor cells, regardless of staining intensity. In formalin-fixed CBs, PD-L1 positivity for all three antibodies ranged from 72.7% to 78.8% at $\geq 1\%$, and from 27.3% to 36.4% at $\geq 50\%$.

All alcohol-fixed CBs—especially those fixed in PreservCyt and CytoLyt—exhibited lower PD-L1 positivity scores than formalin-fixed CBs (42.4%–69.2% vs. 72.7%–78.8% at $\geq 1\%$; 6.1%–30.3% vs. 27.3%–36.4% at $\geq 50\%$). These differences were statistically significant for all fixatives at all cutoff levels for PD-L1 SP263 (Friedman test, $p < .001$ –.023) and for most cutoff levels with PD-L1 28-8

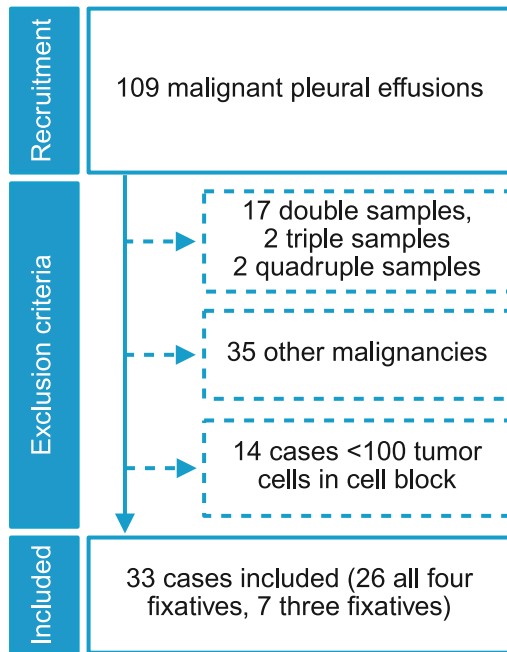


FIGURE 2 Flowchart of the study depicting the collection of malignant pleural effusions with metastatic pulmonary adenocarcinoma on the basis of inclusion criteria from December 2020 to September 2024.

(all Friedman tests were significant on the basis of the 33 cases included when CytoRich Red was excluded). For PD-L1 22C3, the differences were statistically significant only at cutoff levels below 50% (Friedman test, $p = .003-.029$).

The McNemar test confirmed significant differences in positivity between formalin- and alcohol-based fixatives. For PD-L1 28-8, PreservCyt differed from formalin at cutoffs below 50% ($p = .008-.016$), and CytoLyt differed at the $\geq 1\%$, $\geq 5\%$, and $\geq 25\%$ cutoffs ($p = .016-.031$). For PD-L1 22C3, significant differences were seen for PreservCyt at cutoffs below 10% ($p = .004-.031$), and for CytoLyt at cutoffs of $\geq 5\%$ ($p = .016-.031$). For PD-L1 SP263, both PreservCyt and CytoLyt differed significantly from formalin at nearly all cutoffs (all $p < .001-.031$), except at the $\geq 75\%$ cutoff. CytoRich Red showed lower PD-L1 expression but no significant differences were observed compared to formalin at any of the applied cutoff levels ($p = .063-.50$).

Additionally, both PreservCyt and CytoLyt were generally associated with slightly lower scores compared to CytoRich Red for all investigated PD-L1 antibodies, although the differences were not statistically significant. Complete data for all included PD-L1 markers, including expression rates, are presented in Table 2.

Concordance of PD-L1 expression between matched CB preparations

Cytology-cytology correlation of PD-L1 expression with the antibodies 28-8, 22C3, and SP263 was evaluated in matched CBs from 33 MPEs fixed in either formalin, PreservCyt, CytoLyt, or CytoRich

Red (the latter only in 26 cases). The concordance of PD-L1 positivity between matched CB preparations fixed with different fixatives was assessed via multiple analytical methods. In Table 3, OPA, PPA, and NPA are presented (with formalin used as the reference standard for the PPA and NPA calculations), with different cutoff levels of positive tumor cells to define positive staining. Table 3 also includes unweighted Cohen κ values for all investigated PD-L1 antibodies on the basis of pairwise comparisons of all included fixatives.

When focusing on the $\geq 1\%$ and $\geq 50\%$ cutoff levels, when comparing with formalin, the lowest Cohen κ and OPA values were observed for CytoLyt, followed by PreservCyt, with PD-L1 SP263. For the $\geq 1\%$ cutoff, κ was 0.382 and 0.463, and OPA was 67% and 73%, respectively. For the $\geq 50\%$ cutoff, κ was 0.229 and 0.526, and OPA was 73% and 82%, respectively. The corresponding values for CytoRich Red were higher compared with formalin, with a κ of 0.669 and OPA of 85% at the $\geq 1\%$ cutoff, and a κ of 0.698 and OPA of 88% at the $\geq 50\%$ cutoff.

In contrast, for PD-L1 28-8 and 22C3, the lowest Cohen κ and OPA values were observed for PreservCyt, followed by CytoLyt. For PD-L1 28-8, the κ values were 0.522 and 0.57, and OPA values were 76% and 79%, respectively, at the $\geq 1\%$ cutoff. At the $\geq 50\%$ cutoff, both fixatives showed a κ of 0.645 and OPA of 88%. For PD-L1 22C3, the κ values were 0.535 and 0.586, with OPA values of 79% and 82%, respectively, at the $\geq 1\%$ cutoff. At the $\geq 50\%$ cutoff, the κ values were 0.792 and 0.864, and OPA values were 91% and 94%, respectively. In comparison, CytoRich Red showed higher concordance with formalin for both antibodies: for PD-L1 28-8, κ was 0.601 and OPA was 81% at the $\geq 1\%$ cutoff, and κ was 0.898 with OPA at 96% at the $\geq 50\%$ cutoff; for PD-L1 22C3, κ was 0.806 and OPA was 92% at the $\geq 1\%$ cutoff, and κ was 0.906 with OPA at 96% at the $\geq 50\%$ cutoff.

Results from all comparisons and additional data on differences in the concordance of positivity for all PD-L1 antibodies between the various fixatives at different cutoff levels are presented in Table 3.

Detailed data on the agreement of PD-L1 expression between matched CBs and all pairwise fixative comparisons, presented as 2×2 tables at all included cutoff levels, are provided in Table S1.

Table S2 presents additional comparisons from pairwise Wk and Wilcoxon signed-rank tests for all PD-L1 antibodies and fixatives with a seven-tier scale ($<1\%$, 1%–4%, 5%–9%, 10%–24%, 25%–49%, 50%–74%, and $\geq 75\%$). Significantly lower scores were observed for PreservCyt, CytoLyt, and CytoRich Red compared to formalin for all PD-L1 antibodies (Wilcoxon signed-rank test, $p = .003-.01$ for CytoRich Red; $p < .001$ for the others). Scores were also significantly lower for PreservCyt than for CytoRich Red across all PD-L1 antibodies ($p = .008-.014$), and for PreservCyt compared to CytoLyt for PD-L1 22C3 only ($p = .039$). In contrast, CytoLyt scores were lower than those for CytoRich Red only for PD-L1 22C3 and SP263 ($p = .014$ and $.002$, respectively).

Figure 3 shows images highlighting the differences in PD-L1 immunostaining with various antibodies on matched CB preparations fixed in the four different fixatives.

TABLE 2 Frequency of PD-L1-positive reactivity at different cutoff levels in four matched cell block preparations, fixed in different fixatives, from pleural effusions with pulmonary adenocarcinoma.

Biomarker (antibody)	Cutoff, %	Frequency of PD-L1 positivity for fixatives, No. (%)				Friedman test, <i>p</i>	
		Formalin (N = 33)	PreservCyt (N = 33)	CytoLyt (N = 33)	CytoRich Red (N = 26)	CytoRich Red excluded (N = 33)	CytoRich Red included (N = 26)
PD-L1 (28-8)	≥1	24 (72.7)	16 (48.5)	17 (51.5)	14 (53.8)	.002	.002
	≥5	21 (63.6)	14 (42.4)	14 (42.4)	12 (46.2)	.002	.004
	≥10	18 (54.5)	11 (33.3)	13 (39.4)	10 (38.5)	.004	.004
	≥25	14 (42.4)	7 (21.2)	8 (24.2)	7 (26.9)	.002	.019
	≥50	9 (27.3)	5 (15.2)	5 (15.2)	6 (23.1)	.018	.194
	≥75	8 (24.2)	3 (9.1)	5 (15.2)	5 (19.2)	.022	.046
PD-L1 (22C3)	≥1	26 (78.8)	19 (57.6)	20 (60.6)	18 (69.2)	.002	.005
	≥5	24 (72.7)	15 (45.5)	17 (51.5)	14 (53.8)	<.001	.003
	≥10	18 (54.5)	12 (36.4)	14 (42.4)	10 (38.5)	.009	.005
	≥25	14 (42.4)	10 (30.3)	11 (33.3)	7 (26.9)	.039	.029
	≥50	12 (36.4)	9 (27.3)	10 (30.3)	7 (26.9)	.097	.194
	≥75	8 (24.2)	4 (12.1)	5 (15.2)	5 (19.2)	.074	.194
PD-L1 (SP263)	≥1	25 (75.8)	16 (48.5)	14 (42.4)	15 (57.7)	<.001	<.001
	≥5	21 (63.6)	11 (33.3)	12 (36.4)	12 (46.2)	<.001	<.001
	≥10	17 (51.5)	11 (33.3)	10 (30.3)	9 (34.6)	.008	.023
	≥25	14 (42.4)	8 (24.2)	7 (21.2)	7 (26.9)	.008	.01
	≥50	11 (33.3)	5 (15.2)	2 (6.1)	5 (19.2)	<.001	.013
	≥75	7 (21.2)	2 (6.1)	2 (6.1)	2 (7.7)	.007	.007
McNemar test, <i>p</i>							
Biomarker (antibody)	Cutoff, %	Formalin versus PreservCyt (N = 33)	Formalin versus CytoLyt (N = 33)	Formalin versus CytoRich Red (N = 26)	PreservCyt versus CytoLyt (N = 33)	PreservCyt versus CytoRich Red (N = 26)	CytoLyt versus CytoRich Red (N = 26)
PD-L1 (28-8)	≥1	.008	.016	.063	1.00	.50	1.00
	≥5	.016	.016	.125	1.00	.50	.50
	≥10	.016	.063	.125	.50	.50	1.00
	≥25	.016	.031	.25	1.00	1.00	1.00
	≥50	.125	.125	–	1.00	–	–
	≥75	.063	.25	.50	.50	.50	1.00
PD-L1 (22C3)	≥1	.016	.031	.50	1.00	.125	.25
	≥5	.004	.016	.125	.50	.25	1.00
	≥10	.031	.125	.125	.50	.50	1.00
	≥25	.125	.25	.25	1.00	1.00	1.00
	≥50	–	–	–	–	–	–
	≥75	–	–	–	–	–	–
PD-L1 (SP263)	≥1	.004	<.001	.125	.625	.25	.063
	≥5	.002	.004	.125	1.00	.063	.125
	≥10	.031	.016	.375	1.00	.50	.50
	≥25	.031	.016	.25	1.00	.50	.25
	≥50	.031	.004	.25	.25	1.00	.25
	≥75	.063	.063	.125	1.00	1.00	1.00

Abbreviation: PD-L1, programmed death-ligand 1.

TABLE 3 Agreement and performance of immunocytochemical PD-L1 biomarkers at different cutoff levels between four matched cell block preparations, fixed in different fixatives, from pleural effusions with pulmonary adenocarcinoma.

Biomarker (antibody)	Cutoff, %	Statistical analysis	Comparison of positivity (95% CI)					
			Formalin versus PreservCyt (N = 33)	Formalin versus Cytolyt (N = 33)	Formalin versus CytoRich Red (N = 26)	PreservCyt versus Cytolyt (N = 33)	PreservCyt versus CytoRich Red (N = 26)	Cytolyt versus CytoRich Red (N = 26)
PD-L1 (28-8)	≥1	κ	0.522 (0.268 to 0.775)	0.57 (0.314 to 0.825)	0.601 (0.313 to 0.889)	0.818 (0.623 to 1.00)	0.847 (0.646 to 1.00)	0.923 (0.776 to 1.00)
		OPA	76 (59 to 87)	79 (62 to 90)	81 (62 to 92)	91 (76 to 98)	92 (75 to 99)	96 (80 to 100)
		PPA	67 (47 to 82)	71 (51 to 85)	74 (51 to 89)	94 (70 to 100)	100 (72 to 100)	100 (73 to 100)
		NPA	100 (66 to 100)	100 (66 to 100)	100 (60 to 100)	88 (64 to 98)	86 (59 to 97)	92 (65 to 100)
	≥5	κ	0.593 (0.348 to 0.837)	0.593 (0.348 to 0.837)	0.698 (0.438 to 0.957)	0.876 (0.709 to 1.00)	0.843 (0.637 to 1.00)	0.843 (0.637 to 1.00)
		OPA	79 (62 to 90)	79 (62 to 90)	85 (66 to 94)	94 (79 to 99)	92 (75 to 99)	92 (75 to 99)
		PPA	67 (45 to 83)	67 (45 to 83)	75 (50 to 90)	93 (66 to 100)	100 (68 to 100)	100 (68 to 100)
		NPA	100 (72 to 100)	100 (72 to 100)	100 (68 to 100)	95 (74 to 100)	88 (63 to 98)	88 (63 to 98)
	≥10	κ	0.588 (0.341 to 0.835)	0.703 (0.474 to 0.932)	0.698 (0.438 to 0.957)	0.87 (0.696 to 1.00)	0.831 (0.61 to 1.00)	0.917 (0.759 to 1.00)
		OPA	79 (62 to 90)	85 (69 to 94)	85 (66 to 94)	94 (79 to 99)	92 (75 to 99)	96 (80 to 100)
	PPA	61 (39 to 80)	72 (49 to 88)	71 (45 to 89)	100 (70 to 100)	100 (63 to 100)	100 (66 to 100)	
	NPA	100 (76 to 100)	100 (76 to 100)	100 (72 to 100)	91 (71 to 99)	89 (66 to 98)	94 (71 to 100)	
≥25	κ	0.535 (0.265 to 0.806)	0.606 (0.343 to 0.868)	0.742 (0.476 to 1.00)	0.914 (0.748 to 1.00)	0.898 (0.702 to 1.00)	0.898 (0.702 to 1.00)	
	OPA	79 (62 to 90)	82 (65 to 92)	88 (70 to 97)	97 (83 to 100)	96 (80 to 100)	96 (80 to 100)	
	PPA	50 (27 to 73)	57 (33 to 79)	70 (39 to 90)	100 (60 to 100)	100 (56 to 100)	100 (56 to 100)	
	NPA	100 (80 to 100)	100 (80 to 100)	100 (77 to 100)	96 (80 to 100)	95 (75 to 100)	95 (75 to 100)	
≥50	κ	0.645 (0.34 to 0.95)	0.645 (0.34 to 0.95)	0.898 (0.702 to 1.00)	1.00 (1.00 to 1.00)	0.885 (0.665 to 1.00)	0.885 (0.665 to 1.00)	
	OPA	88 (72 to 96)	88 (72 to 96)	96 (80 to 100)	100 (88 to 100)	96 (80 to 100)	96 (80 to 100)	
	PPA	56 (27 to 81)	56 (27 to 81)	86 (47 to 99)	100 (51 to 100)	100 (51 to 100)	100 (51 to 100)	
	NPA	100 (84 to 100)	100 (84 to 100)	100 (80 to 100)	100 (86 to 100)	95 (76 to 100)	95 (76 to 100)	
≥75	κ	0.476 (0.116 to 0.836)	0.716 (0.423 to 1.00)	0.785 (0.506 to 1.00)	0.718 (0.354 to 1.00)	0.708 (0.336 to 1.00)	1.00 (1.00 to 1.00)	
	OPA	85 (69 to 94)	91 (76 to 98)	92 (75 to 99)	94 (79 to 99)	92 (75 to 99)	100 (85 to 100)	
	PPA	38 (13 to 70)	63 (30 to 87)	71 (35 to 92)	100 (38 to 100)	100 (38 to 100)	100 (51 to 100)	
	NPA	100 (84 to 100)	100 (84 to 100)	100 (80 to 100)	93 (78 to 99)	91 (72 to 99)	100 (82 to 100)	
PD-L1 (22C3)	≥1	κ	0.535 (0.265 to 0.806)	0.586 (0.313 to 0.859)	0.806 (0.553 to 1.00)	0.937 (0.817 to 1.00)	0.683 (0.412 to 0.954)	0.755 (0.502 to 1.00)
	OPA	79 (62 to 90)	82 (65 to 92)	92 (75 to 99)	97 (83 to 100)	85 (66 to 94)	88 (70 to 97)	
	PPA	73 (54 to 87)	77 (58 to 89)	90 (69 to 98)	100 (80 to 100)	100 (75 to 100)	100 (76 to 100)	

TABLE 3 (Continued)

Biomarker (antibody)	Cutoff, %	Statistical analysis	Comparison of positivity (95% CI)					
			Formalin versus PreservCyt (N = 33)	Formalin versus Cytolyt (N = 33)	Formalin versus CytoRich Red (N = 26)	PreservCyt versus Cytolyt (N = 33)	PreservCyt versus CytoRich Red (N = 26)	Cytolyt versus CytoRich Red (N = 26)
		NPA	100 (60 to 100)	100 (60 to 100)	100 (56 to 100)	93 (66 to 100)	67 (39 to 86)	73 (43 to 91)
	≥5	κ	0.476 (0.228 to 0.725)	0.57 (0.314 to 0.825)	0.683 (0.412 to 0.954)	0.879 (0.718 to 1.00)	0.772 (0.536 to 1.00)	0.923 (0.776 to 1.00)
		OPA	73 (56 to 85)	79 (62 to 90)	85 (66 to 94)	94 (79 to 99)	88 (70 to 97)	96 (80 to 100)
		PPA	63 (43 to 79)	71 (51 to 85)	78 (54 to 92)	100 (76 to 100)	100 (70 to 100)	100 (73 to 100)
		NPA	100 (66 to 100)	100 (66 to 100)	100 (63 to 100)	89 (66 to 98)	80 (54 to 94)	92 (65 to 100)
	≥10	κ	0.645 (0.405 to 0.885)	0.761 (0.548 to 0.974)	0.698 (0.438 to 0.957)	0.874 (0.705 to 1.00)	0.831 (0.61 to 1.00)	1.00 (1.00 to 1.00)
		OPA	82 (65 to 92)	88 (72 to 96)	85 (66 to 94)	94 (79 to 99)	92 (75 to 99)	100 (85 to 100)
		PPA	67 (44 to 84)	78 (54 to 92)	71 (45 to 89)	100 (72 to 100)	100 (63 to 100)	100 (68 to 100)
		NPA	100 (76 to 100)	100 (76 to 100)	100 (72 to 100)	90 (70 to 99)	89 (66 to 98)	100 (77 to 100)
	≥25	κ	0.742 (0.513 to 0.971)	0.809 (0.606 to 1.00)	0.742 (0.476 to 1.00)	0.93 (0.796 to 1.00)	0.898 (0.702 to 1.00)	1.00 (1.00 to 1.00)
		OPA	88 (72 to 96)	91 (76 to 98)	88 (70 to 97)	97 (83 to 100)	96 (80 to 100)	100 (85 to 100)
		PPA	71 (45 to 89)	79 (52 to 93)	70 (39 to 90)	100 (68 to 100)	100 (56 to 100)	100 (60 to 100)
		NPA	100 (80 to 100)	100 (80 to 100)	100 (77 to 100)	96 (77 to 100)	95 (75 to 100)	100 (80 to 100)
	≥50	κ	0.792 (0.573 to 1.00)	0.864 (0.683 to 1.00)	0.906 (0.728 to 1.00)	0.926 (0.784 to 1.00)	0.898 (0.702 to 1.00)	0.898 (0.702 to 1.00)
		OPA	91 (76 to 98)	94 (79 to 99)	96 (80 to 100)	97 (83 to 100)	96 (80 to 100)	96 (80 to 100)
		PPA	75 (46 to 92)	83 (54 to 97)	88 (51 to 100)	100 (66 to 100)	100 (56 to 100)	100 (56 to 100)
		NPA	100 (82 to 100)	100 (82 to 100)	100 (79 to 100)	96 (78 to 100)	95 (75 to 100)	95 (75 to 100)
	≥75	κ	0.602 (0.267 to 0.938)	0.527 (0.173 to 0.881)	0.885 (0.665 to 1.00)	0.872 (0.626 to 1.00)	0.866 (0.611 to 1.00)	0.866 (0.611 to 1.00)
		OPA	88 (72 to 96)	85 (69 to 94)	96 (80 to 100)	97 (83 to 100)	96 (80 to 100)	96 (80 to 100)
		PPA	50 (22 to 78)	50 (22 to 78)	83 (42 to 99)	100 (45 to 100)	100 (45 to 100)	100 (45 to 100)
		NPA	100 (84 to 100)	96 (79 to 100)	100 (81 to 100)	97 (81 to 100)	95 (76 to 100)	95 (76 to 100)
PD-L1 (SP263)	≥1	κ	0.463 (0.211 to 0.715)	0.382 (0.147 to 0.616)	0.669 (0.387 to 0.951)	0.756 (0.534 to 0.979)	0.772 (0.536 to 1.00)	0.629 (0.357 to 0.90)
		OPA	73 (56 to 85)	67 (50 to 80)	85 (66 to 94)	88 (72 to 96)	88 (70 to 97)	80 (62 to 92)
		PPA	64 (44 to 80)	56 (37 to 73)	79 (56 to 92)	81 (56 to 94)	100 (72 to 100)	100 (68 to 100)
		NPA	100 (63 to 100)	100 (63 to 100)	100 (60 to 100)	94 (71 to 100)	79 (52 to 93)	69 (44 to 86)
	≥5	κ	0.444 (0.205 to 0.683)	0.492 (0.249 to 0.736)	0.698 (0.438 to 0.957)	0.8 (0.585 to 1.00)	0.601 (0.313 to 0.889)	0.683 (0.412 to 0.954)
		OPA	70 (53 to 83)	73 (56 to 85)	85 (66 to 94)	91 (76 to 98)	81 (62 to 92)	85 (66 to 94)

(Continues)

TABLE 3 (Continued)

Biomarker (antibody)	Cutoff, %	Statistical analysis	Comparison of positivity (95% CI)					
			Formalin versus PreservCyt (N = 33)	Formalin versus Cytolyt (N = 33)	Formalin versus CytoRich Red (N = 26)	PreservCyt versus Cytolyt (N = 33)	PreservCyt versus CytoRich Red (N = 26)	Cytolyt versus CytoRich Red (N = 26)
	≥10	PPA	52 (32 to 72)	57 (37 to 76)	75 (50 to 90)	91 (60 to 100)	100 (60 to 100)	100 (63 to 100)
		NPA	100 (72 to 100)	100 (72 to 100)	100 (68 to 100)	91 (71 to 99)	74 (51 to 89)	78 (54 to 92)
		κ	0.64 (0.397 to 0.883)	0.581 (0.33 to 0.831)	0.606 (0.305 to 0.907)	0.651 (0.373 to 0.93)	0.821 (0.586 to 1.00)	0.821 (0.586 to 1.00)
		OPA	82 (65 to 92)	79 (62 to 90)	81 (62 to 92)	85 (69 to 94)	92 (75 to 99)	92 (75 to 99)
		PPA	65 (41 to 83)	59 (36 to 78)	67 (39 to 86)	73 (43 to 91)	100 (60 to 100)	100 (60 to 100)
		NPA	100 (77 to 100)	100 (77 to 100)	93 (66 to 100)	91 (71 to 99)	89 (67 to 98)	89 (67 to 98)
	≥25	κ	0.606 (0.343 to 0.868)	0.535 (0.265 to 0.806)	0.742 (0.476 to 1.00)	0.569 (0.234 to 0.905)	0.785 (0.506 to 1.00)	0.661 (0.321 to 1.00)
		OPA	82 (65 to 92)	79 (62 to 90)	88 (70 to 97)	85 (69 to 94)	92 (75 to 99)	88 (70 to 97)
		PPA	57 (33 to 79)	50 (27 to 73)	70 (39 to 90)	63 (30 to 87)	100 (51 to 100)	100 (45 to 100)
		NPA	100 (80 to 100)	100 (80 to 100)	100 (77 to 100)	92 (74 to 99)	90 (70 to 99)	86 (66 to 96)
	≥50	κ	0.526 (0.224 to 0.828)	0.229 (−0.045 to 0.502)	0.698 (0.391 to 1.00)	0.531 (0.084 to 0.978)	0.598 (0.189 to 1.00)	0.519 (0.069 to 0.968)
		OPA	82 (65 to 92)	73 (56 to 85)	88 (70 to 97)	91 (76 to 98)	88 (70 to 97)	88 (70 to 97)
		PPA	45 (21 to 72)	18 (4 to 49)	63 (30 to 87)	40 (12 to 77)	75 (29 to 97)	100 (29 to 100)
		NPA	100 (82 to 100)	100 (82 to 100)	100 (79 to 100)	100 (86 to 100)	91 (71 to 99)	88 (68 to 96)
	≥75	κ	0.387 (−0.005 to 0.778)	0.387 (−0.005 to 0.778)	0.435 (0.014 to 0.855)	1.00 (1.00 to 1.00)	1.00 (1.00 to 1.00)	1.00 (1.00 to 1.00)
		OPA	85 (69 to 94)	85 (69 to 94)	85 (66 to 94)	100 (88 to 100)	100 (85 to 100)	100 (85 to 100)
		PPA	29 (8 to 65)	29 (8 to 65)	33 (9 to 70)	100 (29 to 100)	100 (29 to 100)	100 (29 to 100)
		NPA	100 (85 to 100)	100 (85 to 100)	100 (81 to 100)	100 (87 to 100)	100 (84 to 100)	100 (84 to 100)

Note: The unweighted κ coefficient is presented as a range from −1 to 1. OPA, PPA, and NPA values are presented as percentages, with formalin cell block preparation as the nonreference standard. Abbreviations: CI, confidence interval; κ, Cohen kappa; NPA, negative percentage agreement; OPA, overall percentage agreement; PPA, positive percentage agreement.

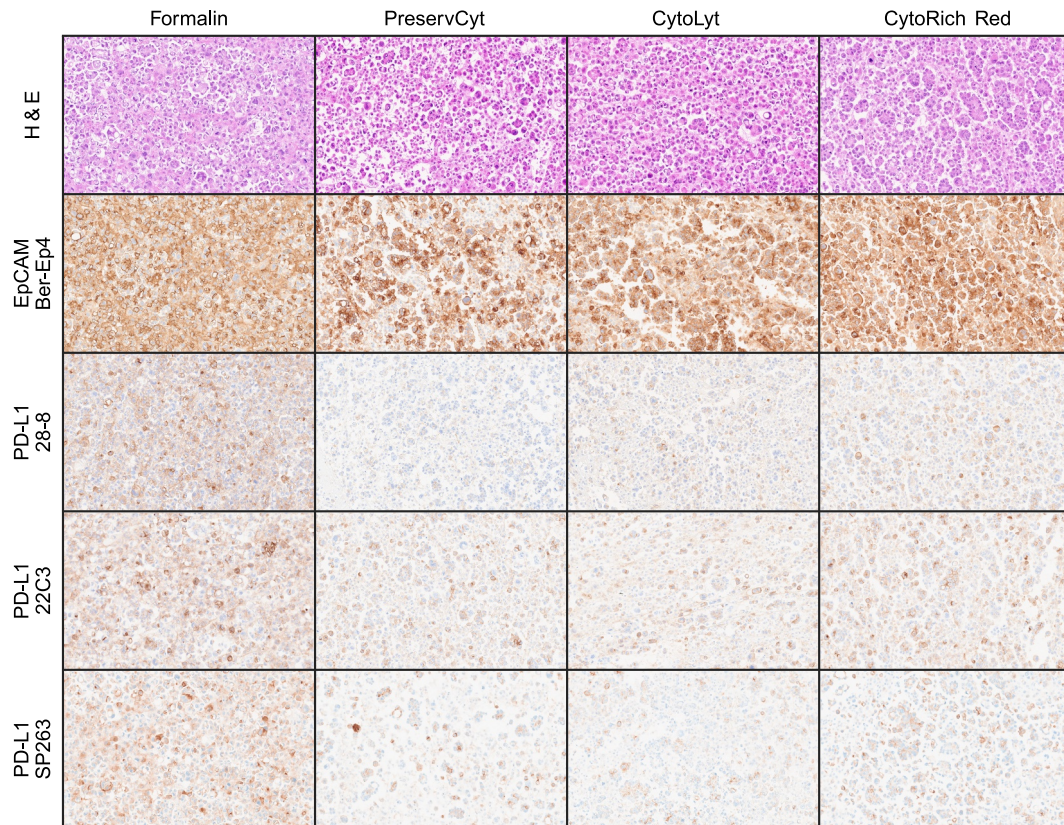


FIGURE 3 PD-L1 reactivity in matched cell blocks with pulmonary adenocarcinoma derived from a single pleural effusion, fixed with different fixatives. H & E staining and immunocytochemical staining with three different PD-L1 antibodies illustrate the discrepancy of PD-L1 expression in alcohol-based fixed cell blocks compared to formalin-fixed cell blocks (original magnification $\times 20$ objective). EpCAM indicates epithelial cell adhesion molecule; PD-L1, programmed death-ligand 1.

Intensity of PD-L1 expression in matched CBs

The distribution of the staining intensity for the PD-L1 antibodies depending on the fixative of the CB is reported in Table 4. As evident, there were significant differences in intensity for all investigated PD-L1 antibodies 28-8, 22C3, and SP263 (Friedman tests, all $p < .001$). For all PD-L1 antibodies, formalin-fixed CBs demonstrated the strongest staining intensity, whereas the lowest intensity varied among the antibodies depending on the specific LBC fixative used. For all PD-L1 antibodies, PreservCyt, CytoLyt, and CytoRich Red were all significantly weaker than formalin (Wilcoxon signed-rank test, $p < .001$ – $.007$). Furthermore, both PreservCyt and CytoLyt were significantly weaker than CytoRich Red for all PD-L1 antibodies ($p = .005$ – $.023$ and $p = .003$ – $.02$, respectively), whereas no significant differences were observed between PreservCyt and CytoLyt for all PD-L1 antibodies. Complete data are presented in Table 4, with additional details, including pairwise W_k values, provided in Table S3.

DISCUSSION

In this prospective study of 33 MPEs with metastatic pulmonary AC, we evaluated the ICC expression of PD-L1 with the antibodies 28-8, 22C3, and SP263 across four matched CB preparations fixed in

formalin, PreservCyt, CytoLyt, and CytoRich Red, respectively. The cytology–cytology correlations of TPS and staining intensity were assessed across multiple cutoff intervals of positive tumor cells. Notable discrepancies in staining properties were observed between fixatives for all evaluated PD-L1 antibodies, particularly at lower cutoff levels, with concordance rates varying by antibody and the cutoff applied. PreservCyt and CytoLyt showed the greatest differences in staining proportions compared to formalin, whereas CytoRich Red demonstrated higher concordance with formalin. Additionally, significant differences in PD-L1 staining intensity were also observed for all alcohol-based fixatives compared to formalin across all PD-L1 antibodies.

Although some reports have indicated no apparent differences between formalin and nonformalin fixation, a handful of studies suggest potential impairment in staining quality. In line with our findings, Koomen et al. used cell lines fixed in different alcohol-based fixatives and observed reduced PD-L1 immunoreactivity compared to those fixed in formalin, which demonstrates that alcohol-based fixation can sometimes negatively affect PD-L1 immunostaining.³⁶ The authors also reported lower concordance in alcohol-fixed/Cel-lient CBs compared to formalin-fixed CBs from ex vivo fine-needle aspiration specimens at both low and high PD-L1 expression levels.³⁶ The deleterious effects of alcohol-based fixation on PD-L1 staining can be partially mitigated by subsequent postfixation in

TABLE 4 Staining intensity in four matched cell block preparations, fixed in different fixatives, from pleural effusions with pulmonary adenocarcinoma.

Biomarker (antibody)	Grading of intensity of cell block preparations, No. (%)															
	Formalin (N = 33)				PreservCyt (N = 33)				CytoLyt (N = 33)				CytoRich Red (N = 26)			
	0	+	++	+++	0	+	++	+++	0	+	++	+++	0	+	++	+++
PD-L1 (28-8)	9 (27.3)	5 (15.2)	16 (48.5)	3 (9.1)	17 (51.5)	11 (33.3)	5 (15.2)	0 (0.0)	16 (48.5)	9 (27.3)	8 (24.2)	0 (0.0)	12 (46.2)	4 (15.4)	9 (34.6)	1 (3.8)
PD-L1 (22C3)	7 (21.2)	5 (15.2)	13 (39.4)	8 (24.2)	14 (42.4)	7 (21.2)	9 (27.3)	3 (9.1)	13 (39.4)	9 (27.3)	9 (27.3)	2 (6.1)	8 (30.8)	7 (26.9)	7 (26.9)	4 (15.4)
PD-L1 (SP263)	8 (24.2)	7 (21.2)	13 (39.4)	5 (15.2)	17 (51.5)	8 (24.2)	8 (24.2)	0 (0.0)	19 (57.6)	8 (24.2)	5 (15.2)	1 (3.0)	11 (42.3)	6 (23.1)	7 (26.9)	2 (7.7)
Biomarker (antibody)	Friedman test, <i>p</i>		Wilcoxon signed-rank test, <i>p</i>													
	CytoRich Red excluded (N = 33)	CytoRich Red included (N = 26)	Formalin versus PreservCyt (N = 33)	Formalin versus CytoLyt (N = 33)	Formalin versus CytoRich Red (N = 26)	PreservCyt versus CytoLyt (N = 33)	PreservCyt versus CytoRich Red (N = 26)	CytoLyt versus CytoRich Red (N = 26)								
PD-L1 (28-8)	<.001	<.001	<.001	<.001	.002	.157	.005	.014								
PD-L1 (22C3)	<.001	<.001	<.001	<.001	.007	.705	.005	.003								
PD-L1 (SP263)	<.001	<.001	<.001	<.001	.005	.405	.023	.02								

Note: Scoring scale (dominating intensity): 0, negative expression; +, weak expression; ++, moderate expression; +++, strong expression.

Abbreviation: PD-L1, programmed death-ligand 1.

formalin; however, this effect is antibody dependent. Unlike PD-L1 SP263, the additional formalin fixation after initial alcohol fixation preserved staining intensity when using PD-L1 22C3.³⁶ Furthermore, another study by Koomen et al. showed that alcohol prefixation of cytological material before formalin fixation can lead to false-negative PD-L1 immunostaining results at both low and high cutoff levels.³⁷ Therefore, we included CytoLyt in the present study for comparison with formalin, given that it is commonly used as both a transport and lysis medium for various cytological specimens. In contrast, Lou et al. demonstrated high concordance between 52 paired CytoLyt- and formalin-fixed CBs; however, they examined the effect of CytoLyt as a prefixative, given that it is the most commonly used nonformalin fixative in clinical practice. Subsequently, the specimens were fixed in formalin before CB preparation.³⁸

As evident from Table 2, only a limited number of cases were PD-L1 positive in non-formalin-fixed CBs at higher cutoff levels. The most recent CAP guidelines recommend including at least 20 positive and 20 negative samples when validating predictive markers.³⁰ The validation set should cover the full clinical spectrum—including high and low expressors—and address expected challenges. This raises the possibility that small fixative-related differences in ICC results may have gone undetected.

In the present study, significant differences were observed at nearly all cutoff levels via the Friedman test, which indicates systematic variation between alcohol-based fixatives and formalin. However, the McNemar test did not reveal any significant differences in pairwise

comparisons at higher cutoff levels for certain PD-L1 antibodies, likely due to insufficient statistical power from the limited sample size to detect such differences. These findings highlight the need for validation of the results in larger cohorts, with balanced representation across no/low/high PD-L1 expression groups—especially in borderline or clinically challenging cases, and across multiple cutoff levels.

Corrections for multiple testing were not applied because of the exploratory nature of the study. Moreover, even with a stricter significance threshold, it is highly unlikely that the results would differ, given the limited number of borderline cases. Nevertheless, the results should be interpreted with some caution because the purpose of the study was exploratory.

As illustrated in Table 3, pairwise comparisons between fixatives showed high variability in OPA between formalin- and alcohol-fixed CBs, which ranged from 67% to 96% (unweighted Cohen κ , 0.229–0.906) depending on the specific fixative and cutoff level applied. The CAP recommends an OPA of at least 90% between test samples,³⁰ with attention to avoiding a skewed distribution of discordant cases. This benchmark assumes an equal distribution of positive and negative cases in the validation set because real-world distributions may lead to misleading conclusions for certain biomarkers and cutoff levels.

The CAP guidelines also highlight the importance of assessing not only the proportion of cells with linear membranous staining but also the staining intensity and overall quality, including background staining.³⁰ As shown in Table 4 and Table S3, all alcohol-based

fixatives resulted in significantly weaker PD-L1 staining intensity compared to formalin across all investigated antibodies (Friedman test, all $p < .001$; Wilcoxon signed-rank test, $p < .001$ – $.007$). In certain cases, alcohol-fixed CBs exhibited weaker and inconsistently diffuse cytoplasmic staining patterns, which made interpretation more challenging. Therefore, immunostaining assessments were initially conducted independently by five observers and supported by correlation with corresponding H & E sections and other relevant immunostains from newly prepared slides of each CB. The most commonly agreed-upon score among observers was selected. In line with findings from a recent multi-institutional international study,³⁹ the lowest interobserver concordance was seen within the 1%–49% TPS range. To address potential discrepancies, data sets containing both primary and alternative scores from some observers for challenging cases were jointly reviewed by two observers (M.S.I.M. and L.P.), and the sixth observer was consulted when needed to resolve discrepancies, reach consensus scores, and minimize potential reporting bias that could influence statistical outcomes. However, a formal analysis of interobserver agreement in PD-L1 evaluation was beyond the scope of this study.

In our study, the significant impact of alcohol-based fixatives on PD-L1 staining proportion and intensity highlights the variability in cytology fixation and CB processing between pathology laboratories. This may partly explain the significant interlaboratory differences in PD-L1 positivity rates^{40,41} and the staining variability observed in cytological specimens reported in the literature.²³ Such inconsistency could potentially lead to inappropriate treatment decisions, especially in borderline cases, where tumors test negative for PD-L1 but might still respond to immunotherapy.

Interpreting the findings of this study requires consideration of its strengths, weaknesses, and limitations. Key strengths include the use of matched and identically prepared and handled cytological CB preparations from the same cases, a systematic approach with multiple fixatives and different PD-L1 antibodies within the same cohort, and a prospective design that minimized methodological differences by eliminating potential confounding factors (e.g., preanalytical variables related to material handling). The fixatives were chosen for their clinical relevance and availability in routine practice. Although the majority of laboratories use formalin-based fixatives or ethanol followed by formalin for CB construction, according to a European survey,²⁶ several studies still report using alcohol-based fixation, as noted in a recent literature review.²³ CytoLyt was included because many cytological specimens are collected, transported, and sometimes stored overnight before processing, especially when CB preparation is requested after initial diagnostic workup. Furthermore, we studied a single tumor type at the same tumor stage, with paired preparations retrieved from the same specimens. All pairs were sampled simultaneously, which ensured they had the same “age” without any time interval between sampling dates, and thereby avoided the influence of biological changes during cancer progression or other confounding factors (e.g., treatment). Previous studies have indicated that PD-L1 expression may be affected if the material is obtained after therapy and suggest that chemotherapy may influence PD-L1 expression in

cancer cells, which potentially skewed the observed outcomes.^{42,43}

We had information regarding treatment: 28 of the 33 cases (85%) were obtained from patients who did not receive any therapy, or the specimens were obtained before any oncological treatment, whereas only five of the 33 cases (15%) were obtained during or after treatment. However, this applied equally to all matched CBs from the same case, which were processed identically with uniform staining and evaluation criteria, which ensured consistent and unbiased results. The primary limitation was the exclusion of many cases due to insufficient material in one or more matched CB preparations (Figure 2), which resulted in a limited sample size, particularly for CytoRich Red, which was not used in the initial cases. Additionally, other relevant fixatives, such as CytoRich Blue, CytoRich Clear, NOVAprep, and TACAS Ruby, as well as mixed or sequential fixation methods (e.g., formalin before, after, or combined with an alcohol-based fixative), were not included. Furthermore, we did not assess fixation duration, cold ischemia time, or sectioning-to-staining time as pre-analytical variables, although these factors were standardized across fixatives within each case. Moreover, we did not correlate the findings with paired histological specimens, although formalin-fixed CBs likely resemble biopsy results, as demonstrated in several studies^{19–23}; however, in this study, 17 cases had paired biopsies, mostly obtained concurrently or within 10 weeks. However, comparisons with biopsies were excluded because we and others have already conducted extensive studies on cytological–histological PD-L1 correlation in non–small cell lung cancer.^{19–23,44} Future studies should incorporate a larger sample size, different fixation strategies (e.g., different fixation times), mixed/sequential fixatives, and additional IHC predictive biomarkers to generate clinically relevant insights.

In conclusion, our data support that the choice of fixative has a demonstrable impact on the immunoreactivity of PD-L1. ICC expression of PD-L1 and its concordance with formalin-fixed CBs vary depending on the type of alcohol-based fixative and the antibody used. Although PD-L1 has previously been reported as a suitable marker for ICC testing on cytological preparations, our study found that all alcohol-based fixatives reduced PD-L1 immunostaining intensity compared to formalin. PreservCyt and CytoLyt were particularly associated with a lower proportion of positive cells across all investigated PD-L1 antibodies. Consequently, false-negative PD-L1 ICC results—especially in PreservCyt- and CytoLyt-fixed CBs—may pose a significant pitfall in PD-L1 assessment, particularly when low cutoff levels are applied. This could lead to incorrect predictive stratification before anti-programmed cell death 1/PD-L1 immunotherapy, which underscores the need to evaluate the reliability of each predictive ICC marker on non-formalin-fixed cytological specimens. ICC protocols should therefore be optimized before applying alcohol-based fixatives in diagnostic settings.

AUTHOR CONTRIBUTIONS

Mohammed S. I. Mansour: Conceptualization, methodology, software, data curation, investigation, validation, formal analysis, funding acquisition, visualization, project administration, resources, writing—original draft, writing—review and editing. **Louise Pettersson:**

Conceptualization, validation, formal analysis, investigation, data curation, visualization, writing—review and editing, funding acquisition. **Rita Pedersen**: Conceptualization, visualization, writing—review and editing. **Ulrich Mager**: Conceptualization, resources, data curation, writing—review and editing. **Kim Hejny**: Methodology, validation, investigation, writing—review and editing. **Frida Kronqvist**: Methodology, investigation, validation, writing—review and editing. **Anna Aronsson**: Methodology, investigation, validation, writing—review and editing. **Tomas Seidal**: Conceptualization, resources, visualization, writing—review and editing, funding acquisition. **Levent M. Akyürek**: Conceptualization, resources, visualization, writing—review and editing. **Ivana Kholová**: Conceptualization, methodology, validation, visualization, investigation, formal analysis, funding acquisition, writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author on reasonable request.

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