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Multiplex plasma protein assays as a diagnostic tool for lung cancer

Mohammad Tanvir Ahamed[1] [iD] | Jenny Forshed[2] | Adrian Levitsky[1] | Janne Lehtiö[2] | Amanj Bajalan[3] | Maria Pernemalm[2] | Lars E. Eriksson[4,5,6] | Björn Andersson[7]

[1]Department of Learning, Informatics, Management and Ethics (LIME), Karolinska Institutet, Stockholm, Sweden

[2]Cancer Proteomics Mass Spectrometry, Department of Oncology-Pathology, Karolinska Institutet, Science for Life Laboratory, Stockholm, Sweden

[3]Department of Microbiology, Tumor & Cell Biology (MTC), Karolinska Institutet, Stockholm, Sweden

[4]Department of Neurobiology, Care Sciences and Society, Karolinska Institutet, Stockholm, Sweden

[5]School of Health and Psychological Sciences, City, University of London, London, UK

[6]Medical Unit Infectious Diseases, Karolinska University Hospital, Huddinge, Sweden

[7]Department of Cell and molecular Biology (CMB), Karolinska Institutet, Stockholm, Sweden

Correspondence
Mohammad Tanvir Ahamed, Department of Learning, Informatics, Management and Ethics (LIME), Karolinska Institutet, Tomtebodavägen 18, Stockholm171 77, Sweden.
Email: tanvir.ahamed@ki.se

Abstract

Lack of the established noninvasive diagnostic biomarkers causes delay in diagnosis of lung cancer (LC). The aim of this study was to explore the association between inflammatory and cancer-associated plasma proteins and LC and thereby discover potential biomarkers. Patients referred for suspected LC and later diagnosed with primary LC, other cancers, or no cancer (NC) were included in this study. Demographic information and plasma samples were collected, and diagnostic information was later retrieved from medical records. Relative quantification of 92 plasma proteins was carried out using the Olink Immuno-Onc-I panel. Association between expression levels of panel of proteins with different diagnoses was assessed using generalized linear model (GLM) with the binomial family and a logit-link function, considering confounder effects of age, gender, smoking, and pulmonary diseases. The analysis showed that the combination of five plasma proteins (CD83, GZMA, GZMB, CD8A, and MMP12) has higher diagnostic performance for primary LC in both early and advanced stages compared with NC. This panel demonstrated lower diagnostic performance for other cancer types. Moreover, inclusion of four proteins (GAL9, PDCD1, CD4, and HO1) to the aforementioned panel significantly increased the diagnostic performance for primary LC in advanced stage as well as for other cancers. Consequently, the collective expression profiles of select plasma proteins, especially when analyzed in conjunction, might have the potential to distinguish individuals with LC from NC. This suggests their utility as predictive biomarkers for identification of LC patients. The synergistic application of these proteins as biomarkers could pave the way for the development of diagnostic tools for early-stage LC detection.

KEYWORDS
confounder correction, diagnostic biomarker, liquid biopsy, lung cancer, machine learning, plasma proteomics, screening

1 | INTRODUCTION

Lung cancer remains one of the most prevalent cancers worldwide, causing 2.2 million deaths per year.[1] The 5-year survival rate for all types of LC is 21%.[1,2] Around 75% of the cases are discovered when the disease is locally advanced (stage III) or metastatic (stage IV), with 5-year survival rates of 13% and 0%, respectively.[3] The symptoms are usually nonspecific and include lasting cough and dyspnea.[4] Though diagnostic tests such as chest radiography, along with computed tomography (CT) and fiberoptic bronchoscopy (FB), are the usual way of LC diagnosis, due to nonspecific symptoms, early-stage patients are not referred to those diagnostic tests for further investigations until they are in later stages of LC.[5-7] LC screening using low-dose CT can reduce LC-specific mortality by 20% compared with screening with chest radiography.[8] However, a high frequency of false-positive results, high cost and the malignancy risk associated with cumulative radiation exposure are limitations of low-dose CT. Therefore, liquid biopsy approaches have garnered considerable attention, as they offer a minimally invasive alternative with lower associated cost and greater accessibility, which may aid in rapid, accurate, and possibly early diagnosis of LC.[9,10]

Histologically, LC is subdivided into two major categories named NSCLC and SCLC. NSCLC constitutes around 85% of the diagnosed LCs, while SCLC is around 15% of cases.[11] The cancer stage at diagnosis and the possibility of surgical treatment are the most crucial factors influencing the survival of LC patients. For stage I, II, and IIIA NSCLC, surgery is the main form of treatment, but for 75% of NSCLCs that are diagnosed at stage IIIB, IIIC, or IV, resection is no longer possible, which increases the mortality risk.[11,12] Therefore, a sensitive and specific method for early detection of LC is essential to improve the survival rate.

Rapid accessibility and minimal invasiveness form the foundation for preferring blood-based assays and plasma biomarkers for LC, which would improve LC detection in both symptomatic and nonsymptomatic individuals.[13-15] Blood-based biomarkers broadly include CTCs, ctDNA, cfDNA, tumor-derived exosomes, TEPs, proteins, and miRNAs.[16-18] The discovery of effective biomarkers (single or in combination) that can be used for referral to other examinations (such as CT scans, bronchoscopy, etc.) for LC, has been difficult due to a lack of analytical accuracy, insufficient patient numbers, and the selection of potential markers. Additionally, the development of biomarkers is often limited because of low statistical power in rare subtypes, making it unlikely that a single marker will be sufficient to diagnose LC. Therefore, it is necessary to explore combinations of markers to improve the accuracy of LC diagnosis.

In this study, we set out to assess plasma samples from a group of patients with primary LC, other cancers, and NC using PEA.[19] PEA offers several advantages over traditional immunoassays, including high specificity for proteins due to the use of DNA-conjugated pairs of antibodies, which minimizes nonspecific binding. These features enable the discovery of disease-specific protein signatures for various diseases and may serve as a promising method for developing protein profiles as potential cancer biomarkers. While this assay is currently used mostly for research purposes, there are studies indicating its potential as a diagnostic tool.[20] The selected 92 immuno-oncology-related proteins in this study were generally involved in tumor immunity, chemotaxis, vascular and tissue remodeling, apoptosis, and tumor metabolism. Although not specifically selected for LC, these proteins are relevant to cancer biology. Among the proteins, we tested which of them, either alone or in combination, were able to classify patients with LC from those with other cancers and NC. Our aim was to develop a set of single or combined proteins as biomarkers for LC that could be used to "flag" patients who may benefit from further diagnostic investigation. Specifically, our focus was on developing "rule-in" biomarker panels to identify patients. Ultimately, the goal was to develop a screening tool that could be used by primary care providers to screen patients with upper respiratory tract and other unspecific symptoms. By identifying these biomarkers, the study aimed to improve the early diagnosis of LC, ultimately leading to better patient outcomes.

2 | MATERIALS AND METHODS

2.1 | Patient sample information

Blood samples were collected from patients with lung inflammatory condition who were referred by primary health care to Karolinska University Hospital (KUH) for investigation for suspected LC during the period between September 2014 and November 2015.[21,22] A cohort of 252 patients was derived from the initial ensemble of plasma donors for the purpose of maintaining data integrity and efficient utilization of available resources (Figure S1). Subsequent to their inclusion in the study, medical records detailing the eventual diagnosis whether LC, other cancer, or NC were meticulously retrieved, ensuring a minimum follow-up duration of 1 year post enrollment. This cohort comprised 173 patients (68.65%) diagnosed with primary LC (indicating the cancer originated in the lungs without any prior cancer history), 56 patients (22.22%) with NC diagnosis, and 23 patients (9.13%) diagnosed with cancers other than LC. Comprehensive demographic and clinical information for all patients ($n=252$) is presented in Table 1. Additionally, detailed clinical diagnostic characteristics for patients diagnosed with primary LC ($n=173$) are presented in Table 2.

2.2 | Plasma sample preparation and PEA

Patient blood samples were collected into potassium EDTA tubes and centrifuged at $3000 \times g$ for 10 min at room temperature within 10–15 min of collection to separate plasma, which was then biobanked at −80°C for future analysis. The Olink multiplex immuno-oncology panel (92 proteins) was applied to these plasma samples. Further information on the Olink PEA method's specificity,

TABLE 1 Clinical information of the patients included in the study ($n = 252$).

	No cancer ($n = 56$)	Lung cancer ($n = 173$)	Other cancer ($n = 23$)
Gender[a]; n (%)			
Female	20 (37.71)	93 (53.76)	10 (43.48)
Male	36 (64.29)	80 (46.24)	13 (56.52)
Age[b]; mean (SD)	65.00 (11.48)	70.06 (8.50)	69.74 (7.61)
Smoking history[c]; n (%)			
Never smoker	18 (32.14)	23 (13.29)	7 (30.43)
Past smoker	25 (44.64)	88 (50.87)	12 (52.17)
Current smoker[d]	13 (23.21)	62 (35.84)	4 (17.40)
Concurrent pulmonary disease[e]; n (%)			
Chronic obstructive pulmonary disease (COPD)	8 (14.29)	34 (19.65)	2 (8.70)
Chronic bronchitis	2 (3.57)	2 (1.16)	0 (0.00)
Pneumonia	17 (30.36)	35 (20.23)	5 (21.74)
Asthma	10 (17.86)	19 (10.98)	2 (8.70)
Pulmonary edema (excess fluid in lung)	6 (10.71)	14 (8.09)	7 (30.43)
Asbestos-related diseases	1 (1.78)	1 (0.58)	1 (4.35)
Emphysema	1 (1.78)	8 (4.62)	0 (0.00)

[a]Distribution of gender does not significantly differ ($\chi 2(2, n = 252) = 5.8$, $p = 0.06$) between the patient groups.

[b]Statistically significant differences exist between the group means as determined by one-way ANOVA ($F(2,249) = 6.61$, $p = 0.0016$). By Tukey's honest significant difference (HSD) method, only the "No cancer vs. Lung cancer" group showed significant ($p = 0.001$) difference.

[c]Distribution of smoking history significantly differed ($\chi 2(2, n = 252) = 13.70$, $p = 0.0083$) between the patient groups.

[d]Current smokers also include those who stopped smoking less than a year ago.

[e]Each patient can have several concurrent conditions.

data validation, NPX value calculation, limit of detection, and reproducibility can be found on the webpage http://www.olink.com.[23,24]

2.3 | Quality control and statistical analysis

2.3.1 | Quality control and sample size

In the course of analyzing NPX values for 92 proteins in patient samples, 12 proteins were omitted owing to inadequate internal quality.

Consequently, the analysis proceeded with samples from 252 patients, focusing on the remaining 80 proteins (Figure S2).

2.3.2 | Exploratory analysis, confounder adjustment, and differential expression

Statistical analysis conducted in this study was of an exploratory nature, as no validated single computational methods have been established for LC-specific biomarker discovery. Hierarchical k-means clustering, and principal component analysis (PCA) were utilized to explore relationships between NPX values and clinical characteristics (LC histology, gender, mutation, cancer stage, smoking status, and concurrent pulmonary disease status) of patients.

Differential protein expression for LC and other cancers versus NC were assessed through univariate analyses. Shapiro–Wilk tests evaluating NPX value normality (non-normality indicated by $p < 0.05$) and Mann–Whitney U tests were utilized for differential expression analysis ($p < 0.05$), incorporating false discovery rate (FDR) correction to mitigate multiple testing issues. To balance true positive detection with false discovery control, the Benjamini–Hochberg procedure was applied for maintaining an FDR of 0.05.

Impact of potential confounding factors (gender, age, smoking status, and concurrent pulmonary disease) were evaluated on NPX values. Patients with at least one pulmonary complication were categorized into the pulmonary disease group ($n = 105$). To assess the impact of confounding variables, associations between NPX and confounders were analyzed for significance ($p < 0.05$) using: Mann–Whitney U test (gender), Spearman correlation (age), Kruskal–Wallis test (smoking status), and Mann–Whitney U test (concurrent pulmonary disease). A linear regression model (LRM) was applied to each protein expression to evaluate the effects of confounding variables on the relationship between NPX and cancer status (LC, other cancer, and NC). Models adjusted for confounders were then compared with unadjusted versions. Confounders that led to a change in effect size of at least 10% in the adjusted LRM were deemed significant and retained. Proteins that remained significant ($p < 0.05$) after adjustment were identified as potential biomarkers. This approach of adjusting for confounders to identify biomarkers was performed over different patient groups based on LC histology, other cancer, and NC.

2.3.3 | Development of discriminative classifier

Based on identified single proteins as potential biomarkers, optimal panel of proteins were developed to distinguish LC and other cancers from NC groups. Furthermore, stratified analyses were performed by considering LC histology (NSCLC, ADC, SCC, SCLC) and stage (early and advanced stage) using both univariate and multivariate approaches. Discriminative classifier was developed using the generalized linear model (GLM) with binomial distribution and logit link function, akin to logistic regression. This method models

TABLE 2 Clinical diagnostics of patients with primary LC ($n=173$).

Cancer type	Histology	Histological subtitle	Ordinal stage[a]	Stage	Total n (%)
Primary LC ($n=173$)	NSCLC (141)	ADC (125)	Early (57)	I	22 (12.72)
				II	10 (5.78)
				IIIA	25 (14.45)
			Advanced (54)	IIIB	8 (4.62)
				IV	46 (26.59)
			Unknown (14)		14 (8.09)
		SCC (32)	Early (21)	I	11 (6.36)
				II	3 (1.73)
				IIIA	7 (4.05)
			Advanced (9)	IIIB	4 (2.31)
				IV	5 (2.89)
			Unknown (2)		2 (1.16)
	SCLC (16)		Early (5)	II	2 (1.16)
				IIIA	3 (1.73)
			Advanced (9)	IIIB	3 (1.73)
				IV	6 (3.47)
			Unknown (2)		2 (1.16)

Abbreviations: ADC, adenocarcinoma; LC, lung cancer; NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma; SCLC, small cell lung cancer.

[a]Stages I, II, and IIIA can be identified as early stages of LC, as tumor does not spread form its primary location. Stages IIIB, IIIC, and IV were identified as advanced stages of LC.

the relationship between a binary outcome (LC, other cancer, or NC) and one or more predictors (proteins). The model's discriminative power was evaluated using AUC, reflecting its ability to differentiate between classes. The diagnostic accuracy of individual protein expression for distinguishing patients with LC or other cancers from NC was measured by AUC. In univariate analysis, proteins with significant differential expression ($p \le 0.05$) and higher AUC values were linearly combined into a panel to achieve an optimized AUC in multivariate analysis. The linear coefficients representing the proteins' contributions to the panel were determined using a nonparametric search by the R package optAUC.[25] The smoothing parameter "$\lambda=5$" and variables (proteins) were standardized by setting scale=T as default arguments in the R function optAUC. The diagnostic effectiveness of both individual proteins and panels of protein was assessed using Youden index (Sensitivity+ Specificity – 1).[26]

3 | RESULTS

3.1 | Potential confounders

Hierarchical k-means clustering was performed using NPX values to assess the relationship between protein expression profiles and clinical characteristics (Figure 1). However, PCA of patient samples formed by NPX values revealed no distinct grouping according to

any clinical characteristics, including LC histology, cancer stage, gender, age, and smoking status (Figure S3).

3.2 | Diagnostic biomarker identification based on single proteins

Univariate analyses were performed to evaluate the differential expression and AUC values of specific proteins among subgroups of patients with LC and other cancers, compared with the NC group.

3.2.1 | Univariate analysis on the primary LC and OC patient groups

Primary LC (n = 173)

Differential expression of individual proteins in patients with primary LC ($n=173$) was evaluated by comparing against the NC group ($n=56$). Post adjustment for confounders, nine proteins (MMP12, CD83, TRAIL, GZMB, CD8A, GZMA, FASLG, CCL19, and CD5) were identified with significant differential expression ($p \le 0.05$) and showed moderate to low diagnostic potential for primary LC, with AUC values ranging from 0.58 to 0.64. Except for MMP12, these proteins were generally expressed at lower levels in the LC group than in the NC group (Table 3; Table S1).
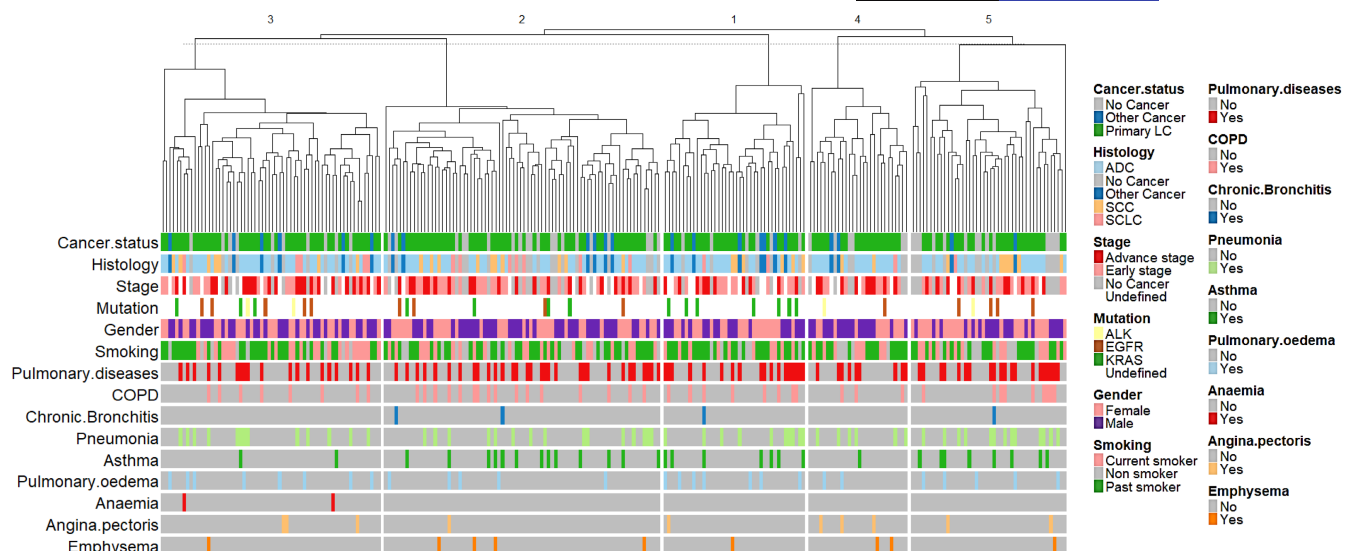
FIGURE 1 Clinical characteristics (LC histology, gender, mutation, stage of cancer, smoking status, and concurrent pulmonary disease status) on clustered plasma protein expression. ADC, adenocarcinoma; LC, lung cancer; SCC, squamous cell carcinoma; SCLC, small cell lung cancer.

Other cancers (n = 23)

The relationship between the expression of individual proteins and other cancers was evaluated by comparing the OC patient group (n = 23) against the NC group (n = 56). Six proteins (CCL20, PDL2, MCP4, MCP2, LAMP3, and LAP TGF beta1) showed significant differential expression ($p \leq 0.5$) and moderate diagnostic potential for the OC group, with AUC values between 0.64 and 0.70. None of these proteins showed significant differential expression between the primary LC and NC group. Additionally, the average expression levels of these six proteins were lower in the OC group compared with both the primary LC and NC groups (Table 3; Table S1).

3.2.2 | Lung cancer histology-based stratified analysis

Patients with NSCLC (n = 157) and SCLC (n = 16)

This study explored the differential expression of proteins among groups of patients with NSCLC (n = 157) and SCLC (n = 16) against NC (n = 56). In the NSCLC cohort, nine proteins (MMP12, CD83, TRAIL, GZMB, CD8A, GZMA, CD4, CCL19, and CD5) and in the SCLC cohort, eight proteins (MMP12, CD8A, IL8, PGF, IL12, CXCL13, FASLG, and CAIX) were identified with significant differential expression ($p \leq 0.05$), showing AUC values ranging from 0.59 to 0.63 for NSCLC and from 0.60 to 0.76 for SCLC. CD8A and MMP12 were the only proteins that demonstrated significant differential expression across both the NSCLC and SCLC groups (Table 3; Table S2).

Patients with ADC (n = 125) and SCC (n = 32)

The NSCLC patient group (n = 157) was further stratified into two subgroups based on histology: patients with ADC (n = 125) and

patients with SCC (n = 32). Protein expressions for both subgroups were compared against the NC group. For the ADC subgroup, eight proteins (TRAIL, CD8A, HO1, CD832, CD5, CCL19, FASLG, and GZMA) showed significant ($p \leq 0.05$) differential expression with AUC values from 0.59 to 0.62. In the SCC subgroup, three proteins (MMP12, TRAIL, and VEGFA) showed significant differential expression with AUC values from 0.62 to 0.74. TRAIL was the only significant protein in both the ADC and SCC subgroups (Table 3; Table S3).

3.2.3 | Lung cancer stage-based (early stage; n = 83 and advanced stage; n = 72) stratified analysis

In early-stage LC patients' samples, 13 proteins (CD83, GZMA, GZMB, CD8A, MMP12, CCL23, TIE2, CSF1, GAL9, PDCD1, CD4, HO1, and PDL2) were significantly differentially expressed ($p \leq 0.05$) when compared with NC patients. PDL2 stood out as the sole protein significantly expressed ($p \leq 0.05$) in patients with other types of cancers. Among these, eight proteins (CD83, GZMA, GZMB, CD8A, MMP12, CCL23, TIE2, and CSF1) were consistently differentially expressed in both early and advanced stages of LC. Furthermore, five of these proteins (CD83, GZMA, GZMB, MMP12, and CD8A) showed significant ($p \leq 0.05$) differential expression across histology (NSCLC, ADC, SCC, and SCLC) and stage-based (early and advanced stage) stratification. (Table 3).

3.3 | Assessment of multiple protein panels

We evaluated the performance of protein combinations as panels to identify potential candidates for guiding LC investigations. A multiprotein panel might serve as an LC screening tool for further in-depth examination and management of suspected patients.

3.3.1 | Selection of protein panels

Protein panels were chosen based on each protein's significant differential expression ($p \leq 0.05$) in the univariate analysis stratified by

histology and stage. This selection process was aimed to compare various LC subtypes and other cancer against the NC group, ensuring the identification of proteins with the highest relevance to malignancy diagnostics and progression. This methodological approach

TABLE 3 Univariate stratified analysis: Significant ($p \leq 0.05$[*]) differential expression of single proteins in different types of LC based on histology and stage.

Panel	Protein	All PLC	Histology NSCLC	Histology ADC	Histology SCC	Histology SCLC	Early PLC	Early NSCLC	Early ADC	Early SCC	Early SCLC	Adv PLC	Adv NSCLC	Adv ADC	Adv SCC	Adv SCLC	Other Cancer
	Sample size (n)	173	157	125	32	16	83	78	57	21	5	72	63	54	9	9	23
	No smoke (n) (%)	23 (13.29)	23 (14.65)	18 (14.40)	5 (15.63)	0 (0.00)	12 (14.46)	12 (15.38)	10 (17.54)	2 (9.52)	0 (0.00)	10 (13.89)	10 (15.87)	7 (12.96)	3 (33.33)	0 (0.00)	7 (30.43)
	Current Smoker (n) (%)	62 (35.84)	53 (33.76)	44 (35.20)	9 (28.13)	9 (56.25)	27 (32.53)	23 (29.49)	18 (31.58)	5 (23.81)	4 (80.00)	26 (36.11)	23 (36.51)	19 (35.19)	4 (44.44)	3 (33.33)	4 (17.39)
	Past Smoker (n) (%)	88 (50.87)	81 (51.59)	63 (50.54)	18 (56.25)	7 (43.75)	44 (53.01)	43 (55.13)	29 (50.88)	14 (66.67)	1 (20.00)	36 (50.00)	30 (47.62)	28 (51.85)	2 (22.22)	6 (66.67)	12 (52.17)
	Pulmonary diseases (n) (%)	75 (43.35)	66 (42.04)	48 (38.40)	18 (56.25)	9 (56.25)	29 (35.90)	28 (35.90)	17 (29.82)	11 (52.38)	1 (20.00)	35 (48.61)	27 (42.86)	22 (40.74)	5 (55.56)	8 (88.89)	15 (65.22)
P1	CD83	▼					▼	▼	▼								
	GZMA	▼	▼	▼			▼	▼	▼			▼	▼	▼			
	GZMB	▼	▼				▼	▼	▼	▼					▼		
	CD8A	▼	▼	▼		▼			▼			▼	▼	▼			
	MMP12	▲	▲		▲	▲				▲		▲	▲	▲	▲	▲	
P2	CCL23										▼	▲	▲		▲		
	TIE2										▼			▼			
	CSF_1						▼	▼	▼					▲			
P3	Gal_9						▼		▼								
	PDCD1								▼								
	CD4		▼						▼								
	HO_1			▼					▼								
P4	TRAIL	▼	▼	▼	▼							▼	▼	▼	▼		
	CD5	▼	▼	▼								▼	▼	▼			
	CCL19	▼	▼	▼								▼	▼	▼			
	FASLG	▼		▼		▼						▼				▼	
	VEGFA				▲							▲	▲	▲	▲	▲	
	IL8					▲						▲	▲	▲	▲	▲	
	CXCL13					▲						▲	▲				
	CAIX					▲										▲	
	PGF					▲										▲	
	IL12					▼										▼	
P5	TNFRSF12A											▲	▲	▲		▲	
	MCP_3											▲	▲	▲	▲		
	FGF2											▲	▲	▲	▲		
	IL7											▲	▲	▲	▲		
	ICOSLG											▼	▼	▼	▼		
	ANG_1											▲	▲	▲			
	IL10															▲	
	CX3CL1															▲	
	CD244													▼			
	MMP7													▲			
	HGF														▲		
	IL6														▲		
	CD40_L														▲		
	MIC_A_B														▲		
	CXCL11														▲		
	TNFSF14														▲		
	EGF														▲		
	CASP_8														▲		
P6	PD_L2								▼								▼
	CCL20																▼
	MCP_4																▼
	MCP_2																▼
	LAMP3																▼
	LAP TGF beta1														▲		▼

Note: Green arrows (▼) indicate lower expression and red arrows (▲) indicate higher expression for the corresponding protein compared with the expression in the group of patients with no cancer.

Abbreviations: ADC, adenocarcinoma; LC, lung cancer; NSCLC, non-small cell lung cancer; PLC, primary lung cancer; SCC, squamous cell carcinoma; SCLC, small cell lung cancer.

[*]Only significant ($p \leq 0.05$) proteins are shown.

was designed to enhance the sensitivity, specificity, and reliability of potential biomarkers for differentiating LC and OC from noncancerous conditions (Table 4).

Panel 1, consisting of five proteins (CD83, GZMA, GZMB, CD8A, and MMP12), showed differential expression ($p \leq 0.05$) within the overall LC cohort ($n = 173$). These proteins were significant across different histological subtypes (NSCLC, ADC, SCC, SCLC) and stages (early and advanced), highlighting their utility as potential LC biomarkers. However, they did not show significance in patients with other cancer types, suggesting their specificity to LC. Specifically, MMP12 had consistently higher expression in LC patients across all subgroups compared with the NC group (Table 3). Early-stage LC showed lower expressions of GZMA, GZMB, and MMP12, while CD83 and CD8A levels were higher—a trend that was inverted in advanced-stage of LC (Figure 2).

Panel 2, consisting of three proteins (CCL23, TIE2, and CSF1), was differentially expressed ($p \leq 0.05$) in early and advanced stages of LC but were not significant in the overall LC cohort, histology, or other cancer type-based strata. Panel 3 comprised four proteins (GAL9, PDCD1, CD4, and HO1), where CD4 and HO1 were differentially expressed across histology and early stages in patients with NSCLC and ADC, respectively. GAL9 and PDCD1 showed significance in early-stage ADC patients. None of these proteins were significant in patient groups of advanced-stage LC or OC types (Table 3). The observed differential expressions of proteins in panels 2 and 3, linked primarily to stage-based and histology/early-stage-based strata, respectively, suggest their potential as biomarkers within specific LC contexts.

Panel 4, containing ten proteins, showed differential expression ($p \leq 0.05$) in four proteins (TRAIL, CD5, CCL19, and FASLG) across the overall LC cohort, with some showing significance in histology and advanced-stage strata but not in the early-stage or OC groups. Panel 5 included 18 proteins that were significantly expressed ($p \leq 0.05$) only in advanced stage of LC, lacking significance in the overall LC cohort, OC group, or in specific histology and early stage of LC (Table 3). The lack of significant expression in early-stage LC for proteins of panels 4 and 5 suggests a limitation in their utility for early detection. Furthermore, expression of these proteins in advanced-stage LC underscores the intricate nature of protein interactions at advanced-stage LC, suggesting a careful consideration for their integration into diagnostic panels.

Panel 6, consisting of proteins (PDL2, CCL20, MCP4, MCP2, LAMP3, and LAP TGF beta1), was differentially expressed ($p \leq 0.05$) in patients with other cancer types, with four proteins (CCL20, MCP4, MCP2, and LAMP3) uniquely identified in this group. Additionally, two proteins, PDL2 and LAP TGF beta1, also showed significance in the LC histology-based groups: PDL2 in early-stage ADC and LAP TGF beta1 in advanced-stage SCC (Table 3). Panels 7, 8, and 9 were developed by different combinations of panels 1, 2, and 3 (Table 4).

### 3.3.2 | Evaluation of protein panels

The classification and diagnostic performance of the selected protein panels in distinguishing LC and other cancer types from NC patients were evaluated using the AUC values and Youden index. This evaluation considered the collective performance of proteins within each panel (Figure 3 and Figure 4).

Protein panel 1 showed strong classification performance within all LC subgroups (AUC: 0.72–0.91) and varying diagnostic

TABLE 4 Proposed panel of proteins.

Panel name	Proteins in the panel	N	Rationale to include in the panel
Panel 1 (P1)	CD83, GZMA, GZMB, CD8A, MMP12	5	• Proteins of this panel were significantly expressed in the full patient cohort and in the histology (NSCLC, ADC, SCC, and SCLC)- and stage (early and advanced stage)-based stratified cohorts
Panel 2 (P2)	CCL23, TIE2, CSF1	3	• Proteins of this panel were significantly expressed in the stage (early and advanced)-based stratified cohorts
Panel 3 (P3)	GAL9, PDCD1, CD4, HO1	4	• Proteins of this panel were significantly expressed in the histology (NSCLC, ADC)- and stage (early ADC)-based stratified cohorts
Panel 4 (P4)	TRAIL, CD5, CCL19, FASLG, VEGFA, IL8, CXCL13, CAIX, PGF, IL12	10	• Proteins of this panel were significantly expressed in the histology- and advanced-stage-based stratified cohorts
Panel 5 (P5)	TNFRSF12A, MCP3, FGF2, IL7, ICOSLG, ANG1, IL10, CX3CL1, CD244, MMP7, HGF, IL6, CD40L, MIC AB, CXCL11, TNFSF14, EGF, CASP8	18	• Proteins of this panel were significantly expressed in the advanced-stage-based stratified cohort
Panel 6 (P6)	PDL2, CCL20, MCP4, MCP2, LAMP3, LAP TGF beta1	6	• Proteins of this panel were significantly expressed in the other cancer patients' group
Panel 7 (P7)	Panel 1 + panel 2	8	• Combined panel of proteins
Panel 8 (P8)	Panel 1 + panel 3	9	• Combined panel of proteins
Panel 9 (P9)	Panel 1 + panel 2 + panel 3	12	• Combined panel of proteins
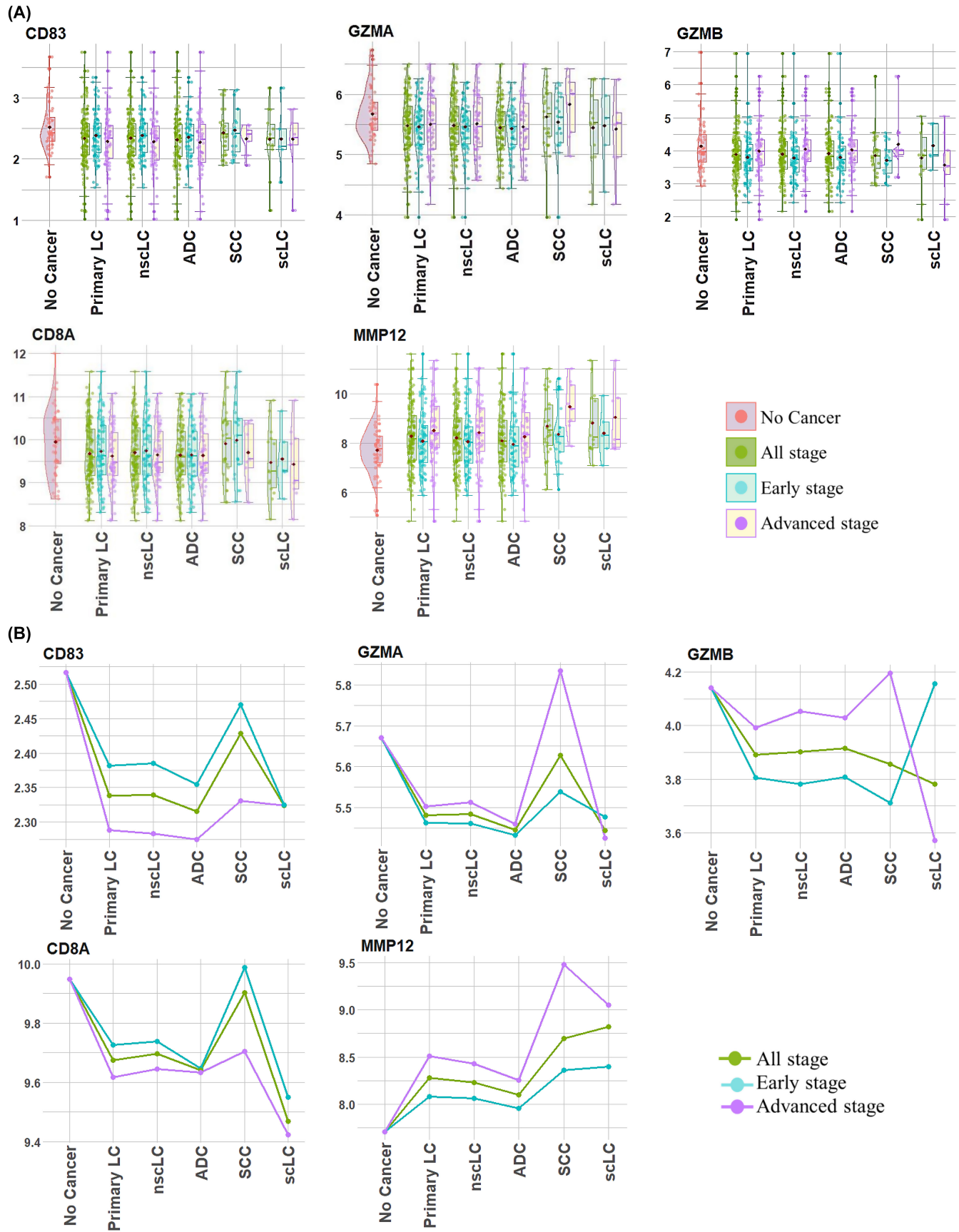
FIGURE 2 Expression of proteins of panel 1 (CD83, GZMA, GZMB, CD8A, and MMP12) in early an advanced stage of different types of LC patients. NPX values on the *y*-axis and different groups of patients based on LC histology in the *x*-axis. (A) Half violin and box plot for panel 1 proteins: The left-side violin plot shows the density of protein expression, while the right-side box plot displays the data distribution across the first (Q1), second (Q2), and third (Q3) quartiles, including outliers. The brown dot represents the mean for each disease group. (B) The mean expressions of individual proteins in different disease groups are shown compared with the no cancer group. ADC, adenocarcinoma; LC, lung cancer; nscLC, non-small cell lung cancer SCC, squamous cell carcinoma; scLC, small cell lung cancer.

(A) Area under ROC curve

(B) Youden Index

(C) Sensitivity
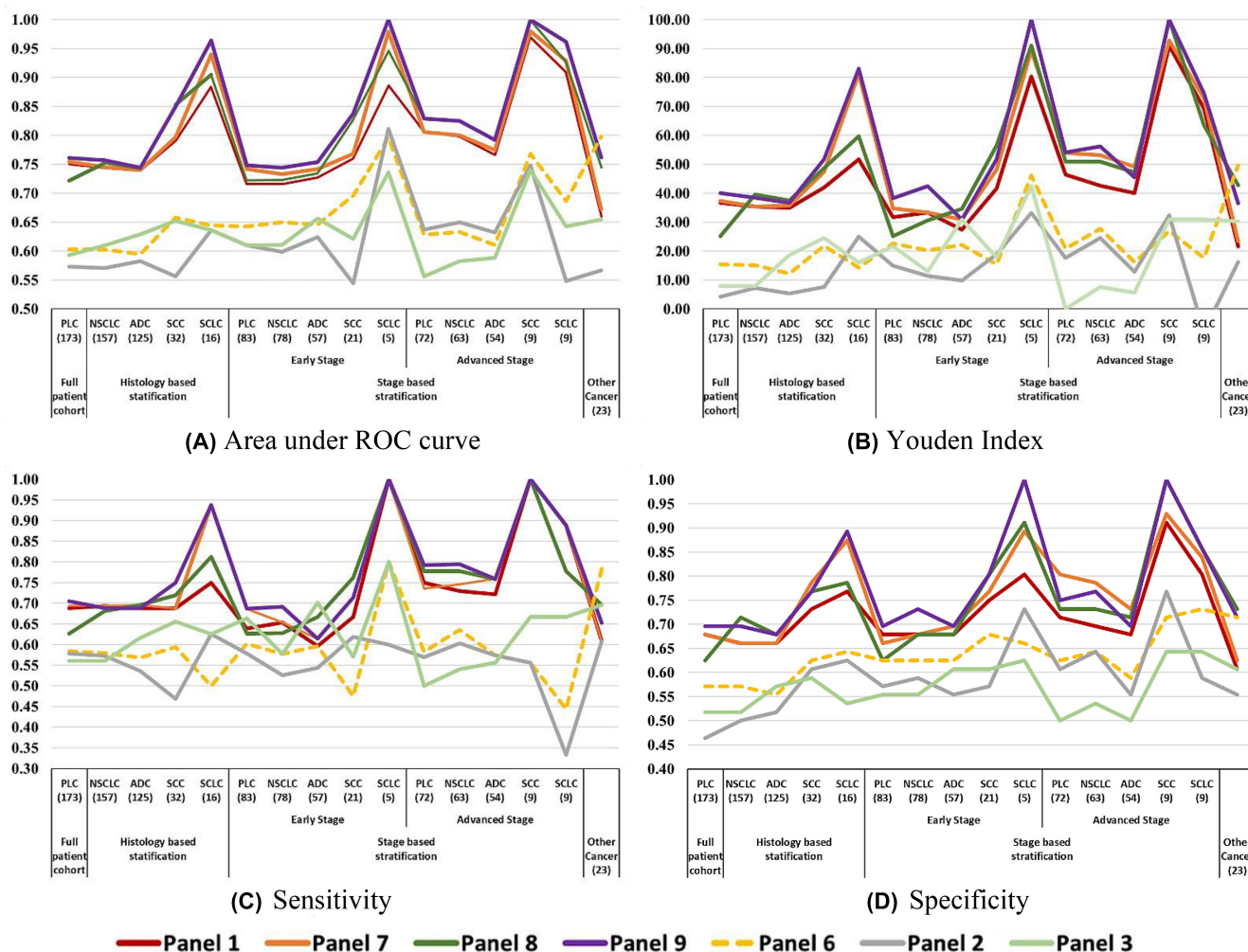
(D) Specificity

FIGURE 3 Classification (AUC) and diagnostic (Youden index) performance for the panel of proteins including their sensitivity and specificity for panels 1, 2, 3, 6, 7, 8, and 9 against the no cancer patient group. (A) Classification performance by AUC. (B) Diagnostic performance by Youden index. (C) Sensitivity. (D) Specificity. For all LC subtypes in different stratified analysis panels 1, 7, 8, and 9 showed higher values in all matric compared to panels 2, 3 and 6. Only for other cancer group, panel 6 showed higher performance in AUC and Youden index. ADC, adenocarcinoma; AUC, area under the receiver-operating characteristic curve; LC, lung cancer; NSCLC, non-small cell lung cancer; PLC, Primary lung cancer; SCC, squamous cell carcinoma; SCLC, small cell lung cancer.

performance (Youden index: 27.5–91.0) across the overall and stratified (histology and stage based) LC patient cohort. Conversely, this panel displayed lower classification and diagnostic performance (AUC: 0.66, Youden index: 21.60) for other cancer types (Figure 3; Table S4).

Panels 2 and 3 showed low classification performance (AUC: 0.54–0.64) across all LC subgroups, except for early-stage SCLC (AUC: 0.81 and 0.74 for panels 2 and 3, respectively) and advanced-stage SCC (AUC: 0.75 and 0.74 for panels 2 and 3, respectively).

The diagnostic performance for each panel was consistently low for all subgroups of patients with LC (Youden index: 4.20–42.50). For the group of patients with other cancer types, panels 2 and 3 also showed low classification performance (AUC: 0.57 and 0.66) and diagnostic performance (Youden index: 16.30, 30.30) (Figure 3; Table S4).
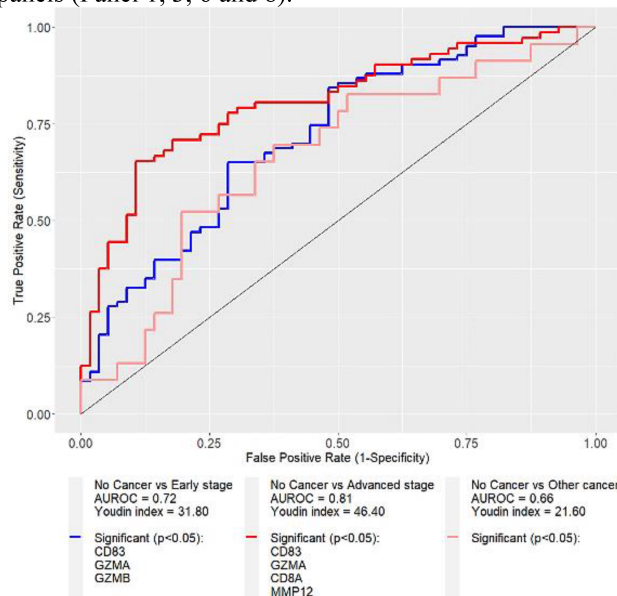
Protein panels 4 and 5 showed moderate to high classification performance (AUC: 0.67–1.00) and varying diagnostic performance (Youden index: 18.7–100.0) across all subgroups of LC. In contrast,
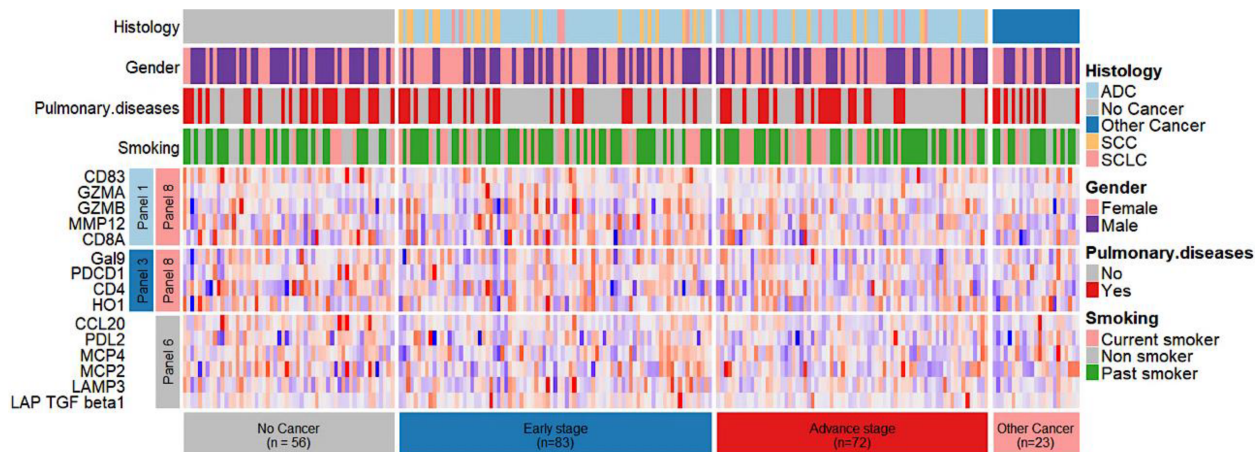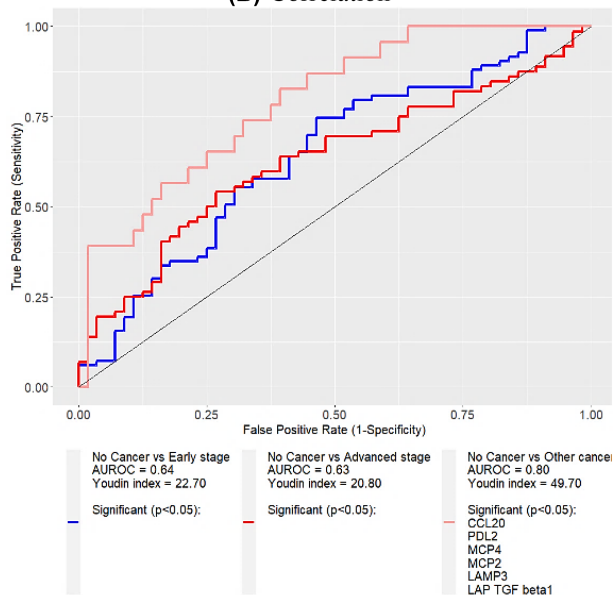
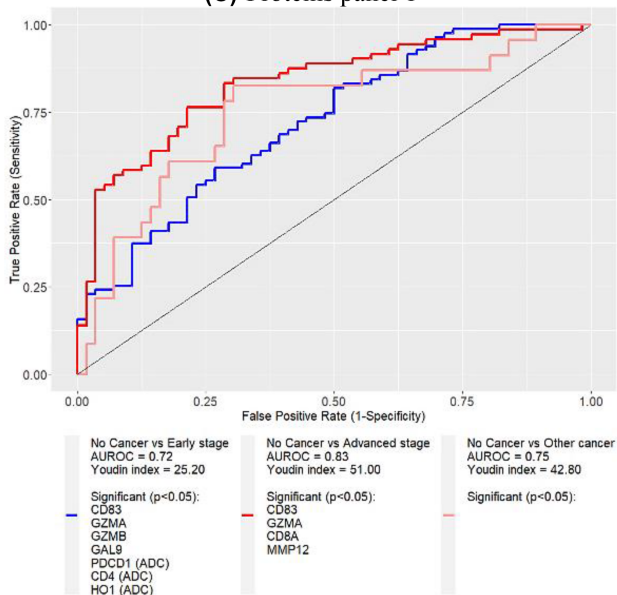**(A)** Expression of proteins in panels (Panel 1, 3, 6 and 8).
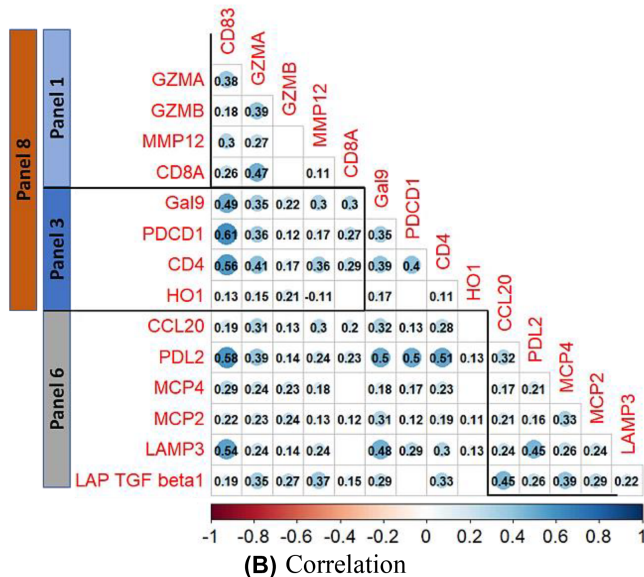
(B) Correlation

(C) Proteins panel 1

(D) Proteins panel 6

(E) Proteins panel 8

FIGURE 4 (A) No grouping tendency was observed in the expression of proteins (panels 1, 3, 6, and 8) in different cancer groups (no cancer, early-stage LC, advanced-stage LC, and other cancer) of patients with different characteristics (histology of LC tumor, gender, status of pulmonary diseases, and smoking). (B) Correlation (Pearson) in the expression of proteins (in panels 1, 2, and 6). The values are the strength of correlation, and insignificant correlations ($p \geq 0.05$ in Pearson's coefficient) are left blank. (C–E) Area under the receiver-operating characteristic curve (AUROC) values and Youden index of panels (panels 1, 6, and 8). Also, single proteins in the panels that are significant ($p \leq 0.05$) between different patient groups (early, advanced, and other cancer) compared with no cancer are demonstrated. ADC, adenocarcinoma; LC, lung cancer; SCC, squamous cell carcinoma; SCLC, small cell lung cancer.

for the group of other cancer types, these panels showed high classification performance (AUC: 0.78 and 0.80) but moderate diagnostic performance (Youden index: 44.60, 46.20) (Table S4).

Panel 6 showed in general low to moderate classification performance (AUC: 0.61–0.80) and diagnostic performance (Youden index: 15.50–46.10) across all LC subgroups. But the performance of this panel was higher for the other cancer types (AUC: 0.80, Youden index: 49.70) compared with both early stage (AUC: 0.64, Youden index: 22.70) and advanced stage (AUC: 0.63, Youden index: 20.80) of LC (Figure 3; Table S4).

When compared with panel 1, panels 7, 8, and 9 showed only slight enhancements in classification (AUC values) and diagnostic accuracy (Youden index) across all LC strata. Additionally, these panels offered modest improvements in sensitivity and specificity also. Nonetheless, they provided notably better performance in identifying other cancer subgroups (Figure 3; Table S4).

4 | DISCUSSION

In this study, we conducted stringent statistical adjustments to address multiple testing issues and carefully considered potential confounding factors, including gender, smoking status, age, and pulmonary disease status. As a result, we successfully pinpointed 46 proteins, constituting 48% of the total proteins examined, which exhibited statistically significant differential expression ($p_{adj} \leq 0.05$) across diverse strata defined by the histology and stage of primary LC and other cancer types when compared with a cohort of patients without cancer. Among these significant proteins, 40 were exclusively identified as significant in the group of patients with primary LC, while 4 were specific to the group of patients with other cancers, and 2 were found in both groups. Furthermore, employing univariate analyses that considered the distinctive expression patterns of several single proteins within different histology and stage-based stratified LC categories, we systematically categorized these 40 proteins identified within the primary LC patient group into five distinct panels: panel 1 (5 proteins), panel 2 (3 proteins), panel 3 (4 proteins), panel 4 (10 proteins), and panel 5 (18 proteins) (Table 4).

Univariate analysis of single proteins' differential expression, conducted on the overall LC cohort and further stratified by histology (NSCLC, ADC, SCC, and SCLC) and stage (early and advanced), revealed that individual proteins in panels 1, 2, and 3 emerged as promising biomarkers for LC diagnostic applications. These proteins in the panel demonstrated unique expression patterns that were distinctively associated with overall LC, histology, and stages, panel 1 with overall LC/histology/stage, panel 2 with stage, and panel 3

with histology and early-stage strata. Conversely, proteins in panel 6 might have potential for diagnosing other cancer types, given their exclusive expression pattern within the other cancer cohort (Table 3).

In multivariate analysis, the collective performance of proteins in panels 1, 2, and 3 was evaluated. Panel 1 (AUC: 0.72–0.91, Youden index: 27.5–91.0) surpassed panels 2 (AUC: 0.54–0.81, Youden index: 4.20–33.20) and 3 (AUC: 0.56–0.74, Youden index: 0.00–42.50) in differentiating various LC histology and stages from the NC group. Panel 6 (AUC: 0.80, Youden index: 49.70) was identified as a promising tool for diagnosing cancers other than LC (Figure 3, Table 3). Subsequently, three more panels (7, 8, and 9) were assessed, showing enhanced capabilities in detecting LC subtypes and other cancers more effectively than panel 1. Among these, panels 7 and 8 had comparable performance in LC detection, yet in identifying cancers other than LC, panel 8 (AUC: 0.75) excelled over panel 7 (AUC: 0.67). Although panel 9 demonstrated superior efficacy to panels 7 and 8, it necessitated a larger set of proteins ($n = 12$) for optimal performance. Thus, panel 8, with its concise protein count ($n = 9$), stands out as the most suitable for LC- and OC-screening applications.

The expression patterns of proteins within panels 1, 6, and 8 (combining panels 1 and 3) showed no dependency with clinical characteristics of patients across various groups (Figure 4A). Additionally, no correlation pattern was observed within or between proteins in panels 1, 6, and 8, highlighting their expression independence (Figure 4B). These characteristics underscore the importance of utilizing multiple proteins together as a panel to navigate the heterogeneity in protein expression. These results suggest that a single protein is insufficient for effectively distinguishing between patient groups, and that a comprehensive approach, analyzing multiple proteins as a combined panel, is essential to reveal unique panel-specific traits.

The findings from this study indicate the utility of panel 1 for LC across all histology and stages (Figure 4C), panel 6 for identifying other cancers than LC (Figure 4D), and panel 8 as a broad-spectrum cancer predictor (Figure 4E), with higher sensitivity and specificity (Figure 3; Table S4). Also, the use of panels significantly improves the performance compared with the individual proteins (Table S5). Moreover, the proteins within panel 1, including CD83, GZMA, GZMB, CD8A, and MMP12, warrant further exploration for their potential roles in cancer biology, highlighting their significance in the diagnosis and understanding of LC.

Membrane CD83 (mCD83), a marker for mature dendritic cells (DCs),[27,28] is also found on activated B cells,[29] T cells,[30] neutrophils,[31] thymic epithelial cells,[32] and even tumor cells,[33] with a

soluble isoform present in serum.[34] It is critical for lymphocyte maturation, activation, and homeostasis, playing a key role in both innate and adaptive immune responses.[35–37] CD83 is involved in T cell stimulation by DCs, whereas its soluble form can inhibit T cell activation.[28,30,38–41] LC patients showed lower DC maturation rates and CD83 expression compared with healthy controls,[42] with our study confirming reduced CD83 levels in LC patients, particularly pronounced in advanced stages (Figure 2). This pattern suggests an initial immune response to early-stage cancer that diminishes as the disease advances, potentially due to the tumor's ability to evade immune surveillance (hallmarks of cancer).

Granzymes (GZMs), serine proteases released by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, are pivotal in immune responses,[43] with GZMA measuring tumor cell cytolytic activity[43–45] and inhibiting tumor cells through the caspase-independent programmed cell death pathway[46] and GZMB inducing apoptosis.[47] Both GZMA and GZMB contribute to NK cell-mediated cytotoxicity toward tumor cells.[48] In LC, GZMA and GZMB may enhance antitumor immunity.[49] This study found higher GZMA and GZMB expression in NC patients, possibly due to robust immune reactions against abnormal cells (hallmarks of cancer) (Figure 2). Importantly, the NC patients in our study had systemic illnesses related to other inflammatory lung conditions that potentially may trigger immune responses resulting in elevated GZM expression. Interestingly, cancer patients exhibited lower GZM levels, likely due to tumor immune evasion.[50] In the current study, advanced-stage LC showed increased GZMA and GZMB expression compared with early stages, suggesting that the higher tumor burden and a more immune-suppressive microenvironment might stimulate immune cell recruitment and activation, leading to elevated GZM levels.

CD8, a coreceptor involved in T cell antigen signaling, plays a vital role in the immune system's fight against cancer, with CD8A indicating the presence of CD8+ cytotoxic T cells.[43,51] Previous studies indicated that CD8A expression is higher in normal lung tissues than in lung ADC and SCC, which also show different levels of myeloid dendritic cell (mDC) infiltration.[52] Current study observed elevated CD8A levels in noncancer individuals and early-stage LC as opposed to advanced-stage LC, implying more effective immune detection and elimination of cancer cells in early stages (Figure 2). Conversely, as LC advances, mechanisms employed by tumors to evade or dampen immune responses (hallmarks of cancer) may lead to decreased CD8A expression.

MMP12, part of the matrix metalloproteinase (MMP) family, plays a critical role in responding to extracellular matrix (ECM) damage and lung protein secretion.[53] Their expression is regulated by various stimuli, including inflammation and hormonal changes, as well as intercellular and matrix interactions.[54] In healthy tissues, MMP expression is low but it increases under pathogenic conditions requiring repair, wound healing, or tissue remodeling (hallmarks of cancer).[55] Previous studies have revealed that elevated expression of MMP12 was associated with local recurrence, metastasis in LC patients, and early cancer-related mortality in NSCLC.[56–59] Smoking

has been shown to elevate MMP12 expression,[60] significant for ECM remodeling,[61] essential in physiological repair processes,[62] and implicated in diseases like emphysema,[63] asthma, Chronic obstructive pulmonary disease (COPD),[64] and LC. Our study found MMP12 expression higher in LC patients than in those without cancer, with increase in advanced-stage LC, aligning with prior findings (Figure 2). Therefore, the inclusion of all five proteins in panel 1 is justified by robust biological evidence and rationale.

In this study, patients in the noncancer group had concurrent inflammatory diseases, and the analyzed proteins were predominantly related to inflammation. Consequently, the low to moderate classification performance of individual proteins in distinguishing SCLC or NSCLC subtypes (ADC and SCC) from NC patients may be influenced by these inflammatory conditions or LC progression, resulting in poor individual protein classification (Table S5). However, when measured collectively as panels, the classification performance for SCLC improves compared with NSCLC subtypes in all panels (Table S4). This improvement may be due to several factors: The higher mutation burden and neoantigen load of SCLC increase baseline immunogenicity[65–67]; SCLC employs immune evasion mechanisms such as MHC class I molecule downregulation and immune checkpoint protein upregulation, unlike the varied strategies in NSCLC subtypes[66,68–70]; and SCLC has a predominantly immunosuppressive microenvironment with elevated regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), compared with the more heterogeneous environments in NSCLC.[66,70–72] Thus, while individual proteins showed low to moderate performance in univariate analysis, their combined use as panels enhances classification by leveraging the integrated protein profile. These findings suggest that combining inflammatory proteins in a panel may significantly enhance the accuracy of LC diagnosis.

The interplay between genomic mutations and inflammatory pathways is crucial in understanding LC progression and the modulation of the identified protein panels. High mutation burdens in LC not only contribute to neoantigen formation and immune activation but also facilitate immune evasion and tumor progression through various mechanisms. These include the alteration of immune checkpoint protein expression, modulation of protein expression levels, and changes in the tumor microenvironment.[73–78] In this study, among the proteins in panel 1, GZMA, GZMB, and MMP12 are directly involved in inflammation, while the remaining proteins play roles in immune regulation, cell signaling, or protective functions. Genomic mutations of immune-related pathways in LC can alter the CD83 regulation,[42,79] affecting DC maturation[27,28] and T cell activation,[30] leading to immune evasion and tumor progression.[35–37] High mutation burdens influence GZMA and GZMB expression, potentially reduce immune response efficacy by inhibiting CTLs and NK cell activity through overexpression of immune checkpoints proteins like PD-L1.[80,81] Conversely, high mutation burdens may enhance CD8A-mediated immune responses through neoantigen formation. However, tumors can develop mutations that enable immune evasion, such as losing MHC class I molecules or upregulating immune checkpoint proteins, hindering the ability of CD8A+ CTLs to target

and eliminate tumor cells, thus contributing to tumor progression.[82] Additionally, genomic mutations in LC can increase MMP12 expression, promoting tumor progression by degrading the ECM and aiding tumor cell migration. Mutations in regulatory pathways can further enhance MMP12 activity, contributing to an aggressive tumor phenotype.[83,84]

Understanding the effects of genomic mutations on the modulation of identified markers provides valuable insights into the tumor biology of LC. This knowledge can inform diagnostic strategies by highlighting the significance of assessing mutation burdens alongside protein expression levels. Additionally, it underscores the potential for developing targeted therapies that address both genetic alterations and inflammatory pathways, improving the efficacy of treatments for LC patients. The identified protein panel, when considered in the context of genomic mutations and mutation burdens, might contribute to the understanding of LC progression. This integrated approach could enhance diagnostic accuracy.

The landscape of LC screening and diagnosis has evolved significantly with the advent of plasma proteomics. A study by Albanes, Demetrius, etal. identifies 36 proteins with potential as biomarkers for early LC detection, where specific proteins like CEACAM5 and MMP12 that showed strong associations with ADC and squamous cell carcinoma, respectively.[85] Another study discusses high-resolution metabolomic biomarkers that distinguish LC patients from healthy controls, emphasizing the role of metabolomics alongside proteomics in LC diagnostics.[86] Another innovative approach that involves the use of superparamagnetic iron oxide nanoparticles (SPIONs) for rapid, deep, and precise profiling of the plasma proteome offers a novel method for biomarker identification.[87] Another study further explores the application of proteomics technologies in cancer liquid biopsies, highlighting the discovery of protein biomarkers via reverse-phase protein array (RPPA) analysis and emphasizing the need for novel protein-based biomarkers in clinical settings.[88] Additionally, a deep learning-based algorithm for LC on chest radiographs showcases the integration of advanced computational methods with traditional diagnostic techniques to improve detection accuracy.[89] Furthermore, another review addresses the global burden of LC, underscoring the importance of advancements in treatment and the potential of proteomics in addressing the gaps in LC care.[90] Collectively, these studies not only demonstrate the potential of plasma proteomics in revolutionizing LC screening and diagnosis but also highlight the importance of integrating these advancements with existing diagnostic methods to enhance early detection and improve patient outcomes.

The promising result of the current study suggests that PEA or similar methods may have potential to detect clinically relevant biomarkers. The ability to rapidly measure multiple plasma proteins simultaneously and identify potential associations between changes in protein expression and LC is a promising area for further research and development.

Differentiating LC from other inflammatory lung conditions poses a significant diagnostic challenge. Including patients without cancer but with inflammatory lung conditions in this study likely enhanced the accuracy of the protein panels' detection capabilities. Although potential protein panels for LC diagnosis were identified, independent cohort validation is necessary to ensure their reliability. The limited sample size and retrospective design of this study necessitate larger, prospective investigations to fully evaluate these protein panels' effectiveness. Furthermore, future studies aimed at validation are vital for the clinical application of these promising panels.

The discovery of proteins suitable for LC screening, especially in early stages, holds significant clinical importance. This study's well-characterized cohort enabled the identification of various proteins, leading to the formation of potential panels that could distinguish between primary LC, OC, and NC patients. Particularly, the protein panels are robust in accurately differentiating primary LC and effectively stratified LC patients by histology (NSCLC, ADC, SCC, SCLC) and disease stage (early and advanced).

While biomarkers for advanced-stage LC are mainly useful for tracking the effectiveness of treatments, early detection of LC can lead to timely interventions, such as surgery, significantly improving survival prospects. Thus, our results have profound clinical implications. The identified protein panel could accelerate clinical decision-making, streamlining the process for referring patients for tissue biopsies, thereby facilitating more accurate diagnoses and improved management of LC patients.

CONSENT

To obtain informed consent, written detailed information about the study along with the invitation to the referred clinical investigation was sent to each patient's home address. Upon the patient's first visit at the thoracic clinic, written informed consent was obtained for both blood sample collection and medical record utilization.

AUTHOR CONTRIBUTIONS

Mohammad Tanvir Ahamed: Conceptualization; data curation; formal analysis; methodology; software; visualization; writing – original draft; writing – review and editing. **Jenny Forshed:** Methodology; writing – review and editing. **Adrian Levitsky:** Data curation; writing – review and editing. **Janne Lehtiö:** Conceptualization; project administration; resources; software. **Amanj Bajalan:** Data curation; formal analysis; methodology; software. **Maria Pernemalm:** Data curation; formal analysis; investigation; writing – review and editing. **Lars E. Eriksson:** Conceptualization; funding acquisition; investigation; project administration; resources; supervision; writing – review and editing. **Björn Andersson:** Formal analysis; methodology; supervision; writing – original draft; writing – review and editing.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest related to this study.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board. Ethical approvals were obtained from Stockholm Regional Ethics Board (EPN: ref no 2014/1290–32).

ORCID

Mohammad Tanvir Ahamed https://orcid.org/0000-0002-5763-2573

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SUPPORTING INFORMATION

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

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