# Circulating levels of Galectin 9 are a potential biomarker predictive of overall survival in patients with advanced Non-Small-Cell Lung Cancer

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**Research Article**

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# Abstract

Background: The immune system is recognized to have therapeutic potential to destroy cancer cells. T-cell immunoglobulin mucin domain-3 (sTIM-3) and its ligand galectin 9 (GAL9) cause suppression of cytokine production, cell cycle arrest, and cell death. Then, soluble levels of TIM-3 and GAL-9 may have prognostic implications in non-small-cell lung cancer (NSCLC) in non-smoker patients.

Methods: This prospective cohort study, including 49 NSCLC patients with an age median of 65 years, evaluated the soluble levels of sTIM-3 and sGAL9 by enzyme-linked immunosorbent assay.

Results: Low levels of sGAL9 in smokers compared to non-smoker NSCLC patients (P<0.0001). Overall survival (OS) analysis showed a low median survival of 10.5 months in smokers NSCLC patients compared to non-smokers (Log-Rank test; P = 0.009). The area under the curve (AUC) value of the Receiver Operating Characteristic (ROC) curve was 0.8497, suggesting that sGAL9 could be an OS biomarker for advanced NSCLC, being the cut-off value of serum of 1,694 pg/mL for sGAL9. Low sGAL9 levels NSCLC patients' group had significantly shorter OS than those in the high sGAL9 levels (Log-Rank test; P = 0.02).

Conclusion: This study indicated the accuracy of sGAL9 as a potential biomarker predictive of OS in advanced NSCLC.


# 1 Introduction

Lung cancer (LC) is the second most frequent type of cancer and the principal cause of death worldwide. Globally, in 2020, around 2.2 million new cases were reported and almost 1.8 million deaths [1]. More than 85% of patients are classified as non-small-cell lung cancer (NSCLC). Its high incidence is strongly related to smoking, implicated in 80%–85% of cases [2, 3]. Cancer immunotherapy by immune-checkpoint has demonstrated a consistent response and increase in overall survival. The therapy with monoclonal antibodies antagonist of the program death-cell 1 (PD1) and its ligand (PDL1) receptors showed well-accepted in LC patients, but not in all patients [4].

New inhibitory targets of the immune system response have been identified with potential anticancer effects. T-cell immunoglobulin mucin domain-3 (TIM-3) is another immune checkpoint as a T-cell activation/exhaustion marker by interferon-$\gamma$ producing T cells when expressed on the cell membrane. The galectina-9 (GAL-9) is a ligand of the TIM-3 receptor. It can stimulate it to regulate the immune response, promote CD8+T cell exhaustion, expand myeloid-derived suppressor cells, and improve the immunosuppressive capacity for tumor progression [5]. The potential clinical of TIM-3 and Gal-9 has been identified in patients with lung cancer from tumor tissue or tumor-infiltrating lymphocytes [6–8].

Cigarette smoking seems to compromise the host inflammation response [9] and induce immune dysfunction in the lungs [10]. Smoking increases the number of alveolar macrophages and stimulates the production of pro-inflammatory mediators, reactive oxygen species, and proteolytic enzymes that could

cause different kinds of tissue damage [9]. Smoking could alter cancer signaling pathways and the tumor microenvironment [10]. When used to identify biomarkers in patients with LC, blood samples could be cost-effective, easily collected, operator-independent, and rapid [11]. The identified biomarker can predict the disease or the evolution process. Higher soluble TIM-3 was associated with poor survival [12].

However, levels of soluble TIM-3 (sTIM-3) and GAL-9 (sGAL-9) in NSCLC need to be evaluated because they can be potential biomarkers and immune-stimulating targets for prognosis or treatment. Thus, in this study, we aimed to assess the expression of sTIM-3 and sGAL-9 in the blood of advanced NSCLC patients.

# 2 Methods

### 2.1 Study Design

This cohort study was performed at Instituto de Medicina Integral Prof Fernando Figueira (IMIP), Recife/PE and Universidade de São Paulo (UNIFESP), São Paulo/SP. The institutional review board of IMIP (No 29195220.1.0000.5505) approved the study protocol. Forty-nine patients were diagnosed with advanced NSCLC from January 2019 to January 2020.

Peripheral blood samples were collected during the patients' initial appointments and those without ongoing cancer treatment. Smoking status was categorized as a non-smoker, defined as patients who had never smoked or ex-smokers who had stopped smoking more than 24 months before the admission date. Smokers are patients who still smoke or had stopped smoking in less than six months before the admission date. Tumor histopathology was estimated according to World Health Organization (WHO) and TNM (tumor, node, and metastasis) staging according to UICC (International Union Against Cancer) classifications [13,14].

### 2.2 Enzyme-linked immunosorbent assay (ELISA)

Expression of soluble TIM-3 (sTIM-3) and sGAL-9 were determined by the enzyme-linked immunosorbent assay (ELISA) method, with commercially available antibodies (R&D Systems, Minneapolis, USA). Absorption was read at 450 nm using an enzyme immunoassay plate reader (BioTek Instruments) with the wavelength correction set to 540 nm.

### 2.3 Statistical Analysis

Data analysis was performed using GraphPad Prism program v8.0. A descriptive analysis was performed using mean and standard deviation, median and interquartile. The Chi-square test and Fischer's exact test were used to compare the distribution of the categorical variables, and any cases with missing data were excluded from the analysis. A non-parametric test was used to compare two groups (Mann–Whitney U test) and t student test for mean and standard deviation analysis. Kaplan–Meier survival curves with log-rank tests were used to estimate overall survival (OS). The receiver operating characteristic (ROC) curves were up to evaluate the predictive value of sGAL-9 and sTIM-3. The sGAL9 and sTIM3 ROC were

performed in NSCLC patients who died versus survivors. The specific cut-off for GAL9 was calculated by ROC analysis. Differences were considered statistically significant when *P* values were < 0.05.

# 3 Results

## 3.1 Clinical variables

This analysis included 49 patients with advanced NSCLC with an age median of 65 years and were 26 men (53.1%) and 23 women (46.9%). Twenty-three smokers (47%) and 26 non-smokers (53%). The latter had a more significant smoking load: 64.2% smoked more than 30 packs/year. The adenocarcinoma type was the most frequently diagnosed (45.0%), followed by undifferentiated (28.5%) and squamous cell carcinoma (26.5%). Forty-two patients (85.7%) were stage IV, and 14.3% were stage III (Table 1).

**Table 1.** Characteristics of the 49 patients with non-small cell lung cancer (NSCLC)

| **Variables** | **N=49** | |
|---|---|---|
| **Age** | | |
| Median (IQR) | 65.0 (54.0-75.0) | |
| | **N** | **%** |
| **SEX** | | |
| Male | 26 | 53.1 |
| Female | 23 | 46.9 |
| **HISTOLOGY TYPE** | | |
| Adenocarcinoma | 22 | 45.0 |
| Squamous Cell Carcinoma | 13 | 26.5 |
| Undifferentiated | 14 | 28.5 |
| **TNM** | | |
| III | 7 | 14.3 |
| IV | 42 | 85.7 |
| **SMOKERS** | | |
| Yes | 23 | 47.0 |
| No | 26 | 53.0 |

TNM: tumor-node-metastasis system; IQR: interquartile

### 3.2 sGAL9 and sTIM-3 levels in NSCLC patients

Low levels of sGAL9 in smokers compared to non-smoker NSCLC patients ($p<0.0001$). There were no significant differences in sGAL-9 levels between age ≤ 65 > 65 years, sex, and histopathology types (**Figure 1**). There were no significant differences in sTIM-3 levels between smokers and non-smoker, age ≤ 65 > 65 years, sex, and histopathology types (**Figure 2**).

### 3.3 Overall survival in NSCLC patients

Overall survival (OS) analysis showed a low median survival of 10.5 months in smokers NSCLC patients compared to non-smokers (Log-Rank test; *P = 0.009*; Figure 3).

By using the ROC curve, we found that a baseline of 1694 pg/ml (cutoff). sGAL9 with specificity (72.2%), sensitivity (83.2%), and area under the curve - AUC=0.8497 (*P<0.0004*) for differentiating the median overall survival in advanced NSCLC (**Figure 4**). Low sGAL9 levels NSCLC patients' the group had significantly shorter OS, a median survival of 15 months, than those in the high sGAL9 levels (Log-Rank test; *P = 0.02*). AUC sTIM3 were of 0.64, were not statistically significant in the advanced NSCLC (Figure 5).

## 4. Discussion

Thus, this study showed that changes in sGAL-9 levels were associated with smoking and shorter overall survival in NSCLC. Galectins contribute to carcinogenesis and cancer progression, including cancer, proliferation, metastasis, angiogenesis, and the immune response [15].

This study also showed high sGAL9 and better OS in non-smokers NSCLC patients than smokers. Tobacco smoke contains many chemical and carcinogenic particles that could develop chronic pulmonary inflammation and lung cancer. Curiously, the lung cancer of smokers and non-smokers have distinct inflammatory signatures, with marked differences in mast cell and CD4+ T cell numbers. Furthermore, high regulatory T cells, even at early stages, and reduced CD8+ T cells in the tumor lesion; have been related as an immune escape mechanism in NSCLC patients [10].

High sGAL9 levels were associated with a better prognosis in OS analysis compared to the patients' group with low sGAL9 levels. Thus, we can infer that smoking not only could damage immune system processes due to chronic pulmonary inflammation, with reduction of sGAL9 levels but also favors the onset of LC and its worse prognosis. A limited number of studies have investigated the role of GAL9 in lung cancer. Increased GAL9 expression in tumor cells may suppress pulmonary metastasis, recurrence of melanoma, and breast cancer [16].

Kadowaki et al. 2013 shown in lung carcinoma cell-bearing mice that GAL9 expression induces macrophage differentiation into plasmacytoid dendritic cell-like macrophages, which may augment the

activation of NK cells and was associated with, increased the survival of tumor-bearing mice [17]. Only 15%–20% of NSCLC patients under immune checkpoint inhibitor treatment achieve a partial or complete response. The resistance mechanism to immunotherapy needs to be better-understood [18]. One study showed that early accumulation of monocytic myeloid-derived suppressor cells expressing GAL9 [18].

The analysis of expression of GAL-9 is on NSCLC tumor cells, and tumor-infiltrating lymphocytes (TILs) showed that low GAL9 levels on tumor cells or high GAL-9 levels on TILs were more likely to have a poor prognosis. GAL-9 on TILs correlated with TIM-3, PD-1, and PD-L1 levels, and on tumor cells, GAL-9 was associated with the expression of TIM-3 [19]. D'Alessandro et al. 2020 demonstrated that Galectins 1, 3, and 9 levels have been changed in serum idiopathic pulmonary fibrosis, suggesting their potential utility as clinical, diagnostic, and prognostic biomarkers [20].

In NSCLC patients, high TIM-3 and GAL-9 levels correlated with larger tumor sizes, advanced stages, and more distant metastasis [21]. Lymphoid cells with high TIM-3 expression are associated with primary and secondary resistance in metastatic LC patients under anti-PD-1 treatment [21]. Moreover, TIM-3 is often co-expression with PD1 in exhausted CD8 cells in infections and tumors, which could explain why an isolated blockade of TIM-3 could be effective [21].

This study indicated the predictive accuracy of sGAL9 in the survival advanced NSCLC. We suggest that changes in sGAL9 levels could be associated with smoking and deaths in lung cancer. However, an increase of GAL9 levels in the serum of non-smokers patients was associated with a better OS in advanced NSCLC. In conclusion, sGAL9 is a potential biomarker predictive of OS in advanced NSCLC.

## Declarations

# Author Contributions Statement

**Author contributions**: All authors have participated directly and wrote the manuscript, approved the version to be published, and agreed to be accountable for all aspects of the work. **Guilherme Jorge Costa**: Conceptualization; investigation, methodology, data curation, formal analysis, funding acquisition, data interpretation, and writing - original draft. **Guilherme Vieira de Mendonça Filho**: Investigation, writing - original draft, and data curation; **Leuridan Cavalcante Torres**: Investigation, methodology, formal analysis, funding acquisition, visualization, resources, and writing - review & editing; **Dulce Elena Casarini**: Supervision, project administration, investigation, data curation, and writing - review & editing.


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**CONFLICT OF INTEREST:** None of the authors has any potential financial conflict of interest related to this manuscript.

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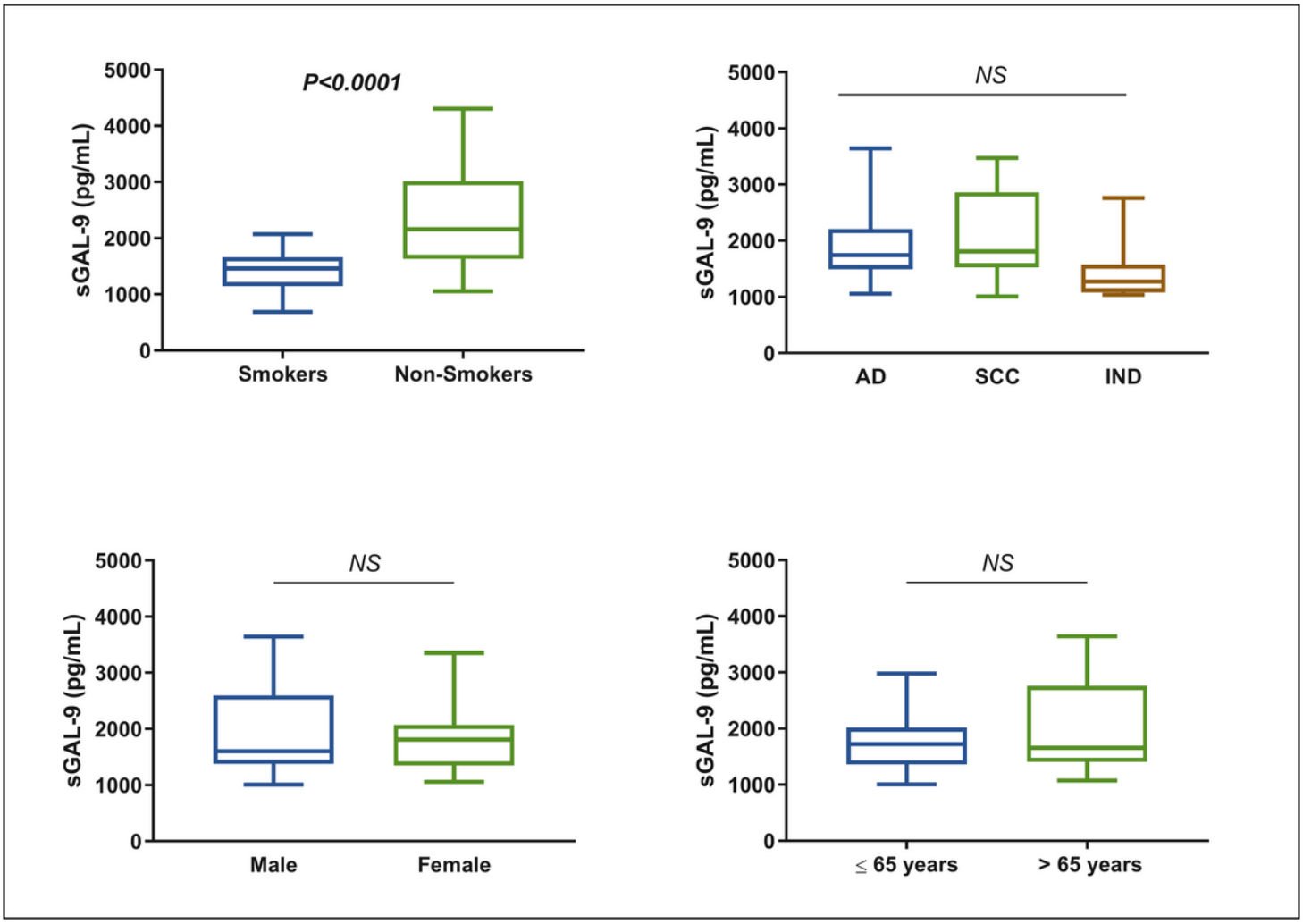
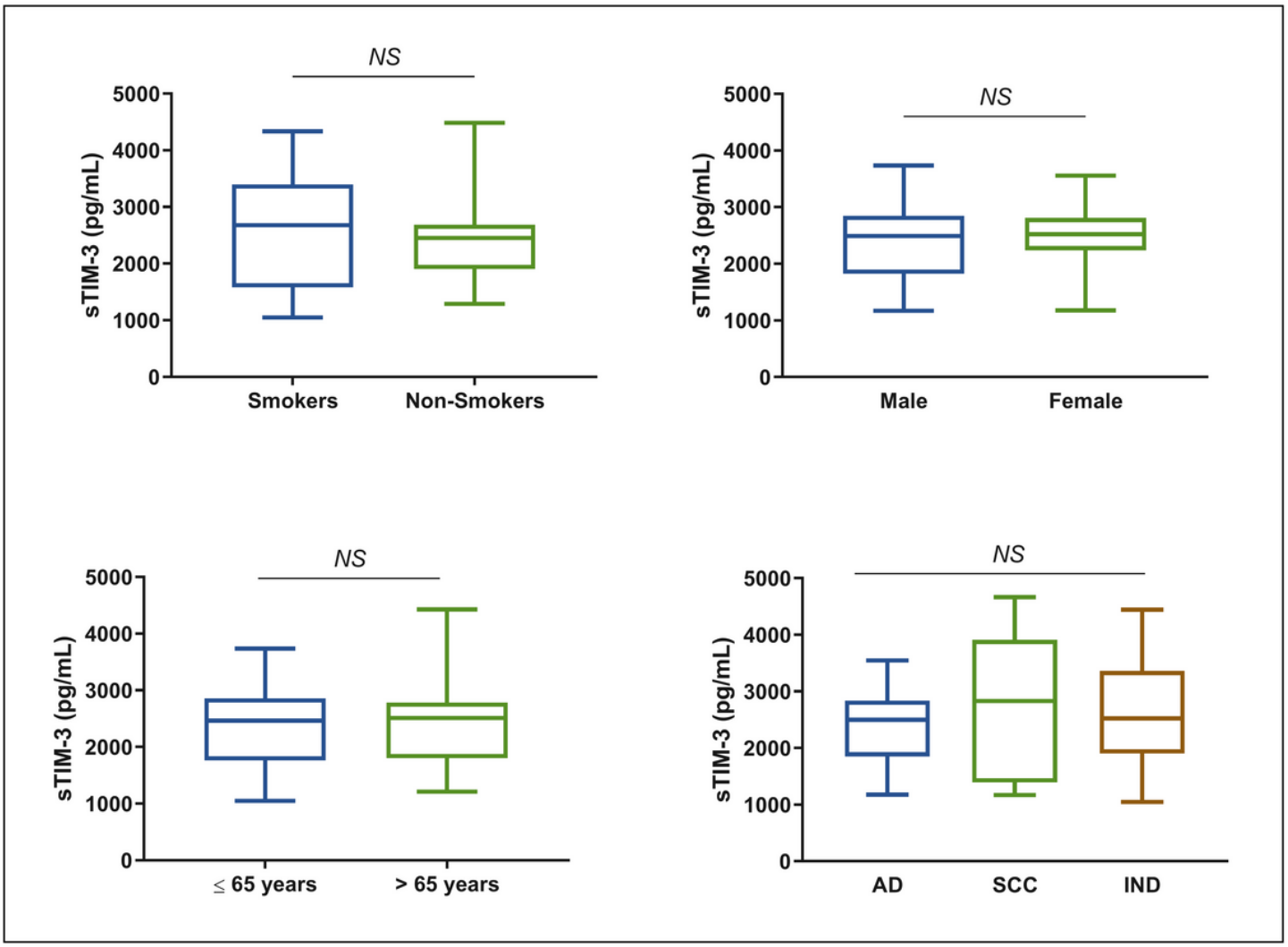
# Figures



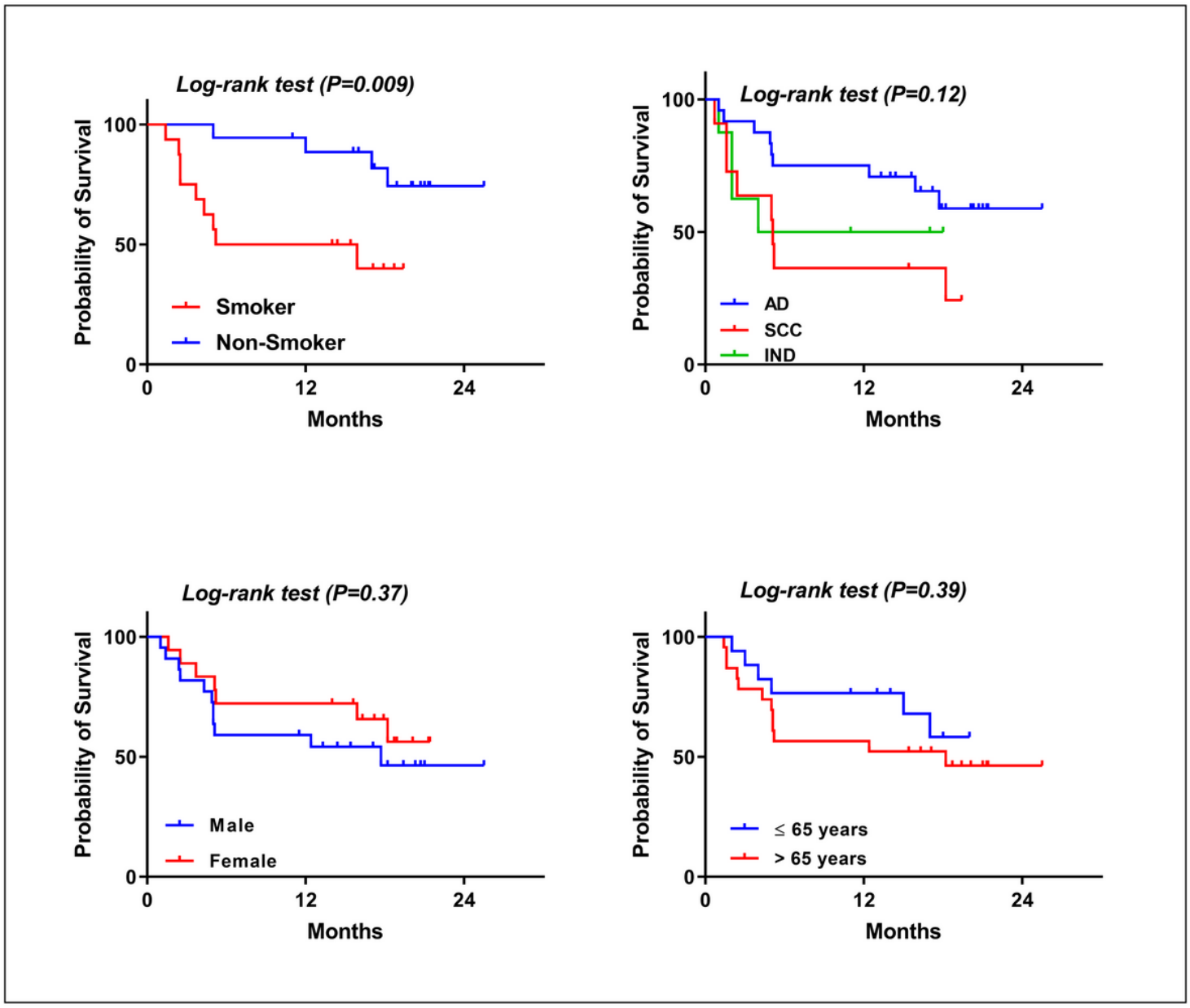

## Figure 1

sGAL-9 levels in advanced NSCLC patients, accordingly, smoking status, age, sex, and histopathology types. The graphs are represented in median and interquartile. The Mann Whitney test was performed. It was considered significant $p<0.05$. NS: Not significant. Adenocarcinoma (ADD), Squamous cell Carcinoma (SCC) and Undifferentiated (UND).



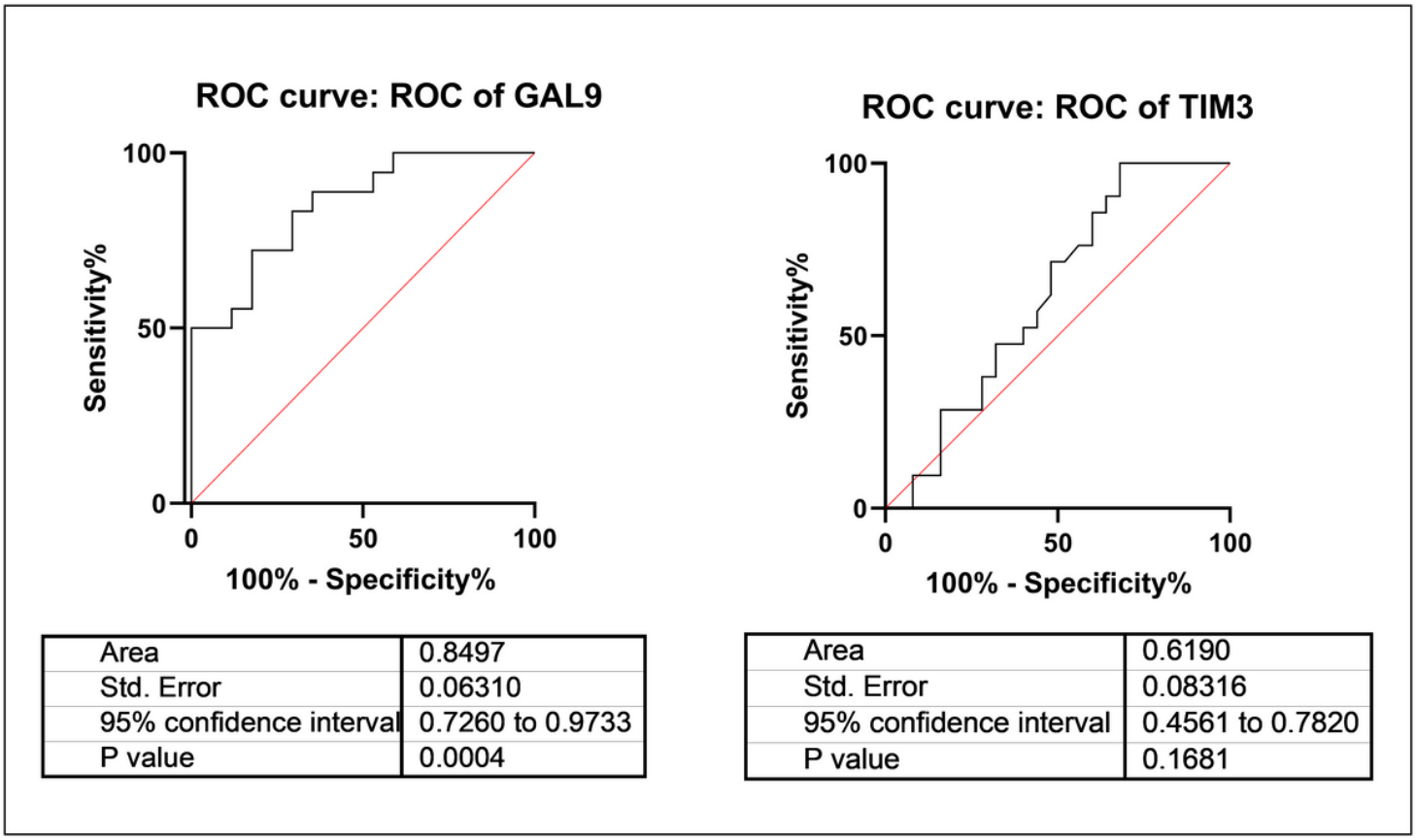

## Figure 2

sTIM3 levels in advanced NSCLC patients, accordingly, smoking status, age, sex, and histopathology types. The graphs are represented in median and interquartile. The Mann Whitney test was performed. It was considered significant $p<0.05$. NS: Not significant. Adenocarcinoma (ADD), Squamous cell Carcinoma (SCC) and Undifferentiated (UND).




## Figure 3

The association between smoking status, age, sex, and histopathology types with overall survival of NSCLC patients. Adenocarcinoma (ADD), Squamous cell Carcinoma (SCC) and Undifferentiated (UND).




| | |
|---|---|
| Area | 0.8497 |
| Std. Error | 0.06310 |
| 95% confidence interval | 0.7260 to 0.9733 |
| P value | 0.0004 |

| | |
|---|---|
| Area | 0.6190 |
| Std. Error | 0.08316 |
| 95% confidence interval | 0.4561 to 0.7820 |
| P value | 0.1681 |

## Figure 4

ROC analysis evaluating prognosis accuracy of serum sGAL9 and sTIM3 in advanced NSCLC.

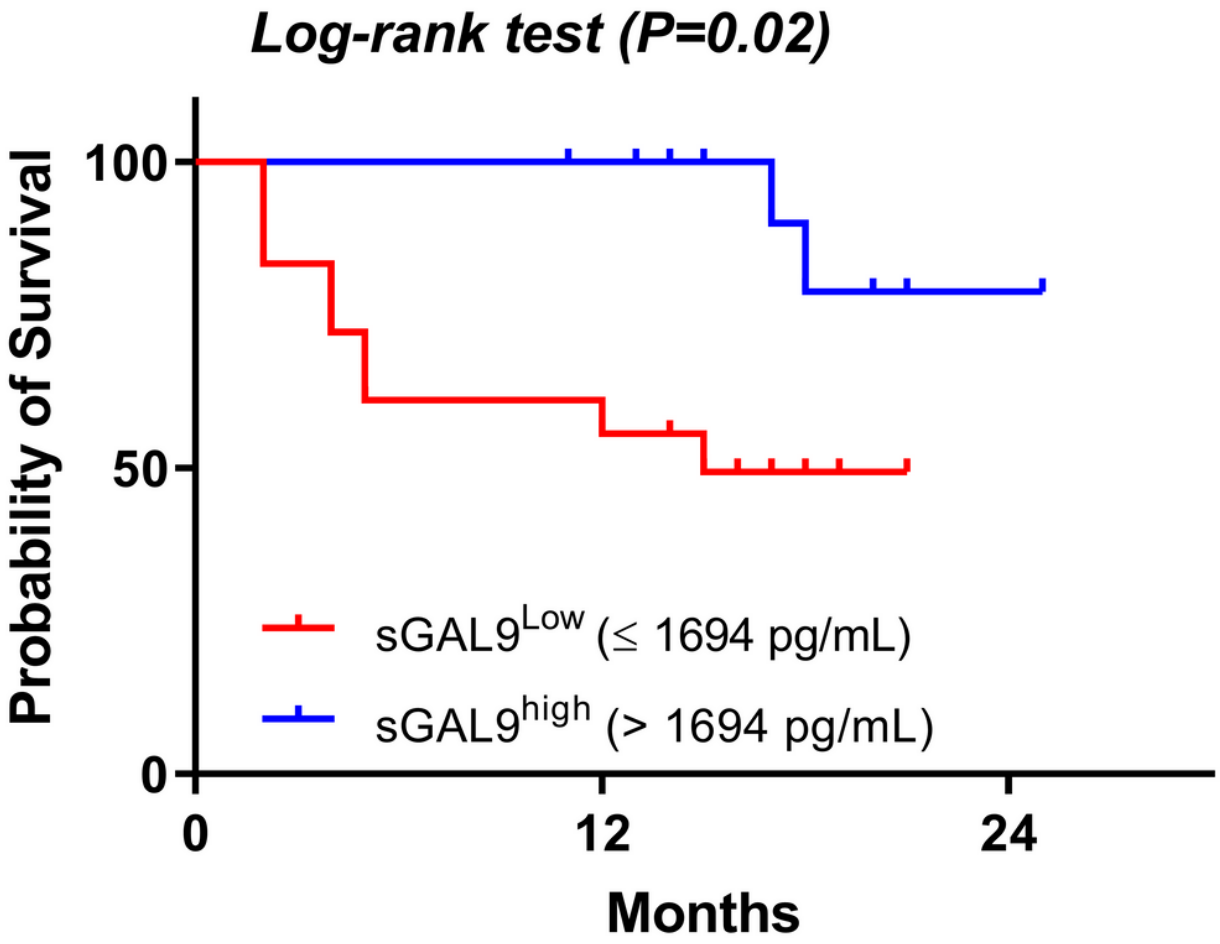



**Figure 5**

Prognostic effects of sGAL9 levels on overall survival of advanced NSCLC patients.