DOI:10.14753/SE.2025.3227

SEMMELWEIS EGYETEM

DOKTORI ISKOLA

# Ph.D. értekezések

# 3227.

# SZENTKERESZTY MÁRTON

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DOI:10.14753/SE.2025.3227

# Inflammation and immune response in non-small cell lung cancer

**PhD thesis**

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Budapest
2025

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## List of abbreviations

| | |
|---|---|
| ADC | Adenocarcinoma |
| ALK | Anaplastic lymphoma kinase |
| ANA | Antinuclear antibody |
| ANC | Absolute neutrophil count |
| ANOVA | Analysis of variance |
| BMI | Body mass index |
| CI | Confidence interval |
| COPD | Chronic obstructive pulmonary disease |
| CRP | C-reactive protein |
| CT | Computed Tomography |
| CTLA4 | Cytotoxic T-lymphocyte associated protein 4 |
| ECOG | Eastern cooperative oncology group |
| EGFR | Epidermal growth factor receptor |
| ELISA | Enzyme-linked immunosorbent assay |
| FCS | Fetal Calf Serum |
| $FEV_1$ | Forced expiratory volume in 1 second |
| FFPE | Formalin-fixed paraffin-embedded |
| FoxP3 | Forkhead box P3 |
| FVC | Forced vital capacity |
| G-MDSC | Granulocytoid myeloid-derived suppressor cell |
| GOLD | Global initiative for chronic obstructive lung disease |
| granz-$B^+$ | Granzim-B positive |
| Hb | Hemoglobin |
| HR | Hazard ratio |
| ICS | Inhaled corticosteroid |
| IFN-γ | Interferon-gamma |
| IL-10 | Interleukin-10 |
| IL-2 | Interleukin-2 |
| KRAS | Kirsten rat sarcoma virus oncogene homolog |
| LABA | Long-acting β adrenoceptor agonist |
| LAMA | Long-acting muscarinic antagonist |

| | |
|---|---|
| Ly | Absolute lymphocyte count |
| MDSC | Myeloid-derived suppressor cell |
| MEM | Minimum Essential Medium |
| MET | Hepatocyte growth factor receptor |
| M-MDSC | Monocytoid myeloid-derived suppressor cell |
| Mo | Absolute monocyte count |
| MRI | Magnetic Resonance Imaging |
| NE | Neutrophil elastase |
| Neu / Ly ratio | Neutrophil-to-lymphocyte ratio |
| NK cell | Natural killer cell |
| NKG2D | Activating receptor belonging to the NKG2 family |
| $NK^{high}Treg^{high}$ | Both NK and Treg cell density above median |
| NKp46 | Natural cytotoxicity triggering receptor 1 |
| NS | Not significant |
| NSCLC | Non-small cell lung cancer |
| OS | Overall survival |
| PBMC | Peripheral blood mononuclear cell |
| PD-1 | Programmed cell death protein 1 |
| PD-L1 | Programmed death-ligand 1 |
| PET | Positron Emission Tomography |
| PFS | Progression-free survival |
| PI3K | Phosphoinositide 3-kinases |
| PMA | Phorbol 12-myristate 13-acetate |
| RB1 | Retinoblastoma 1 |
| RECIST | Response evaluation criteria in solid tumors |
| RFMD | Restriction fragment microfluidic based detection |
| ROS1 | Receptor tyrosine kinase c-ros oncogene 1 |
| SCC | Squamous cell carcinoma |
| SCLC | Small cell lung cancer |
| SEM | Standard error of the mean |
| STK11 | Serine / threonine kinase 11 |
| TCGA | The cancer genome atlas |

DOI:10.14753/SE.2025.3227

| | |
|---|---|
| $T^{exh}$ cell | Exhausted T-cell |
| TGFβ | Transforming growth factor beta |
| TIL | Tumor-infiltrating lymphocytes |
| TME | Tumor microenvironment |
| TNF-α | Tumor necrosis factor alfa |
| TP53 | Tumor protein P53 |
| Treg cell | Regulatory T-cell |
| VEGF | Vascular endothelial growth factor |
| WBC | White blood count |
| $\chi^2$ test | Chi-squared test |

## 1. Introduction

Lung cancer is the leading cause of cancer-related mortality on a global scale, which is indicative of its widespread prevalence as a result of tobacco smoking (1). The mortality and incidence rates have typically been highest in high-income countries, particularly the European countries and the USA. In Hungary, men are diagnosed annually with an average of 5,573 new lung cancers, 5,135 colon cancers, and 3,968 prostate cancers, whereas women are diagnosed with an average of 7,521 new breast cancers, 4,120 colon cancers, and 3,759 lung cancers. Nevertheless, lung cancer is the most common cause of cancer-related mortality in Hungary for both men and women, with an average of 5,425 cases per year and 3,285 cases per year, respectively (2). Most primary lung cancers are carcinomas, mesenchymal and hematolymphoid tumors are less common. The most prevalent histological types are adenocarcinoma (ADC), squamous cell carcinoma (SCC), small cell lung cancer (SCLC), and large cell carcinoma (LCC). Preinvasive lesions, benign epithelial tumors, and other tumors can also occur, however they are less frequent (3). Cigarette smoking has been linked to lung cancer through well-established pathways. Cigarette smoke contains about a hundred recognized carcinogens and is known to cause proinflammatory and mutagenic effects in the lungs (4). Lung adenocarcinomas are known to arise from a series of molecular alterations. Early modifications in preneoplastic lesions, such as atypical adenomatous hyperplasia and adenocarcinoma in situ, have been identified to involve mutations in the EGFR (5) and KRAS (6) genes, as well as loss of heterozygosity affecting several tumor suppressor genes (7). The pathogenesis of lung adenocarcinomas is frequently linked to two simoltaneously altered growth signaling pathways: the p53/RB1/p14/STK11 growth inhibitory pathway and the EGFR/RAS/PI3K growth pathway (8). Approximately 50% of lung adenocarcinomas exhibit inactivating mutations in the TP53 tumor suppressor gene. These mutations are commonly linked to smoking and involve specific genetic changes known as G>T and C>A transversions. Around 20% of lung adenocarcinomas exhibit activating mutations in the KRAS gene, which are also associated with smoking (9). Women, individuals who have never smoked, and people of Asian heritage have a higher probability of having activating mutations in the EGFR gene. Specifically, 70% of never-smokers from eastern Asia, 40% of non-smokers of European origin, and 11% of European smokers had these mutations (10). Rearrangement of ALK and other genes such as MET and ROS1, are only responsible for

a small portion of adenocarcinomas. There is a lack of apparent ethnic disparities, and individuals with these tumors tend to be younger compared to the rest of adenocarcinomas (11). Squamous cell carcinoma arises by a process called multistep transformation, characterized by the gradual accumulation of genetic and epigenetic abnormalities and the development of phenotypic dysplasia. The development of squamous cell carcinoma is strongly associated with disruptions in the genome, genetic mutations, and changes in the expression of important molecules that play a role in the squamous cell differentiation (12). Over the past two decades, many breakthrough discoveries have changed the diagnostic and therapeutic strategies in thoracic oncology. Historically, the critical stages that influenced treatment decisions were the simple distinction between non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC), and the evaluation of the disease's extent. Surgical resection is the most effective curative treatment for NSCLC in the early phases (I-IIIA). Relapse occurs following curative treatment, attributable to variables similar to those in advanced NSCLC. Gender, age, pathological stage and performance status are among the numerous factors that influence progression-free survival (PFS) and overall survival (OS) following resection (13).

In addition, certain serum variables such as cytokines and inflammatory markers, along with blood cells and their ratios, have been shown to be prognostic indicators in NSCLC (14, 15). Profound investigations of surgically removed NSCLC tissues have demonstrated that a high concentration of several immune cell types invading the tumor ($CD3^+$, $CD4^+$, $CD8^+$ T lymphocytes and B cells) is also indicative of a longer survival (16, 17). The tumor microenvironment (TME) often exhibits significant immunosuppression due to the presence of immune checkpoint ligands, such as PD-L1, on tumor cells and other cells within the TME. The interaction between these ligands and the PD-1 receptor of effector T-cells can lead to a state of anergy in effector cells expressing the receptor. Furthermore, regulatory T cells (Tregs) also gather and have the ability to suppress the immune response of $CD4^+$ and $CD8^+$ T cells (18), as well as NK cells (19). The role of antitumor $CD8^+$ T cells in the immune response against cancer progression is well-established. The immune checkpoint inhibitor anti-PD-1 and anti-PD-L1 antibodies, which are often employed in clinical settings, can alleviate the inhibition of effector T cells that express the PD-1 receptor (20). The presence of NK cells infiltrating tumors has been linked to improved prognosis in various malignancies (21-

23), including NSCLC (24-26). NK cells may potentially be stimulated by anti-PD1 antibody therapy, as PD1 is expressed on their surface (27).

Previous immunohistochemical investigations utilized the CD56 or CD57 markers, which are not completely exclusive to natural killer (NK) cells. Recent studies have shown that the presence of $NKp46^+$ cells, identified by the NK-specific marker NKp46, is associated with increased density of tumor-infiltrating cells. This has been observed in certain types of cancers, such as GI adenocarcinomas, GISTs. Furthermore, this increased density of $NKp46^+$ cells has been linked to longer survival rates in certain tumor types (28, 29). The incidence of cancer formation and metastasis was higher in mice lacking NK cells (30). However, the effectiveness of NK cell infiltration alone in fighting malignancies may not be sufficient, as NK cells found within tumors have been observed to have low levels of activating receptors and reduced functional activity, particularly in cases of lung cancer (31).

It is important to mention that the tumor microenvironment (TME) contains numerous cellular and molecular elements that can hinder the ability of NK cells to fight against tumors (32, 33). $CD4^+CD25^+FoxP3^+$ regulatory T cells are a well-studied type of immune cells that have the ability to decrease immunological responses. Accumulation of mainly $FoxP3^{high}$ effector Tregs was observed in the majority of cancers (34). Tumor-infiltrating Tregs' prognostic significance, however, is debatable and greatly varies depending on the kind of tumor. While higher Treg infiltration was linked to a shorter survival time in certain malignancies, other studies found the opposite or found no correlation with survival (21, 35, 36). For instance, it was discovered that high levels of Tregs were linked to good prognoses in cases of head and neck (37), colorectal (38), and breast cancer (39). Regarding NSCLC, the findings are inconclusive, showing a correlation between high Treg density and either unfavorable (40, 41) or favorable prognosis (42-44), or no significant correlation (45-49). According to multiple investigations, the prognostic influence differed among patient subgroups with distinct histological types or smoking status, and it also relied on where Treg cells were located (stroma or tumor islets) (41, 42, 46, 50, 51).

In advanced stage (IIIB/IV) non-small cell lung cancer treatment with cytotoxic and/or biologically targeted drugs during induction and maintenance therapy is the gold standard for advanced NSCLC. Tumor- and host-related variables both play a role in determining

prognosis. Major tumor-related factors include primary tumor stage, lymph node status, and distant metastases, whereas host-related aspects include performance status and weight loss (52). Pain, weight loss, performance state and functional impairment are symptoms of tumor-induced inflammation (53). Increased ANC reflects severity of tumor-induced inflammation, additionally, lower than median absolute neutrophil cell counts (ANC) have been shown to be a strong positive prognostic marker of longer PFS in advanced NSCLC during first-line treatment (54). Conversely, T cell exhaustion (55) and decreased total lymphocyte (Ly) cell counts (56) indicate a poor prognosis in advanced disease. Also the Neu/Ly ratio has been consistently demonstrated to be a reliable predictor of OS and PFS in advanced NSCLC (57, 58). Inflammation, lymphoid dysfunction, VEGF, myeloid-derived suppressor cells (MDSCs), and the development of exhausted T-cells ($T^{exh}$) are also linked to COPD (59, 60), not just cancer. Circulating vascular endothelial growth factor (VEGF) concentration increases in advanced NSCLC (61-63). VEGF has been demonstrated to not only promote angiogenesis, but also to have an impact on myelopoiesis, namely in the development of MDSCs. These cells are known to contribute to both immunosuppression and tumor-induced inflammation (64, 65). The development of so-called exhausted T lymphocytes ($CD4^{+}T^{exh}$ and $CD8^{+}T^{exh}$) in tumor tissue (66) as well as in systemic circulation (67, 68) is one sign of immunosuppression in cancer.

Despite the fact that advanced NSCLC mostly affects heavy smokers and older individuals, many of whom have other medical conditions (69), insufficient information exists about the impact of comorbidities on PFS both during and after initial treatment, one notable exception is the recent demonstration that diabetes mellitus reduces PFS in this state (70). The impact of chronic obstructive pulmonary disease (COPD), the second most common comorbidity after cardiovascular disease, has only been examined in a limited number of previous studies (71-74). A small retrospective study (n=50) documented no change in PFS after first-line treatment in patients with COPD (71), however, OS was observed to either remain unaltered (72, 73) or be prolonged by the presence of concomitant COPD in advanced NSCLC (74). Since almost half of NSCLC patients have COPD (69, 75), a condition characterized by chronic inflammation and impaired lymphoid function, the issue is still clinically significant (76).

DOI:10.14753/SE.2025.3227

## 2. Objectives

The following objectives were established during my doctoral research:

- In our retrospective study of early-stage (I-IIIA) surgically treated lung adenocarcinoma patients (n=115), we analyzed the density of NKp46$^{+}$ NK-cells and FoxP3$^{+}$ Treg-cells within the tumor (stromal and tumor islet) and their impact on progression-free survival (PFS) and overall survival (OS).
- We also investigated how KRAS mutational status and specific clinical variables affect NK and Treg cell infiltration.
- In our prospective study of advanced stage (IIIB-IV) non-small cell lung cancer patients, we investigated the impact of concomitant COPD on tumor-induced inflammation and immune response.
- We also investigated how concomitant COPD influences PFS and OS in advanced stage NSCLC patients following first-line combined chemotherapy.

# 3. Methods

## 3.1. Patients

### 3.1.1 Early stage surgically resected pulmonary adenocarcinoma cohort

Patients diagnosed between 2004 and 2008 with early-stage adenocarcinoma were selected from those who had diagnostic procedures performed at Department of Pulmonology Semmelweis University and surgery performed at Department of Thoracic Surgery Bajcsy-Zsilinszky Hospital, Budapest. Upon careful review of the histological results and sections, we have incorporated all cases of adenocarcinoma (n=115) that occurred between 2004 and 2008. The follow-up period lasted for 60 months. The retrospective study obtained ethical permission from the Institutional Ethics Committee of the Semmelweis University Clinical Center (#238-2/2015). Progression-free survival (PFS) was calculated as the time from the date of surgery to disease progression or death. Overall survival (OS) was estimated from from the date of surgery until exitus.

### 3.1.2 Advanced stage non-small cell lung cancer (NSCLC) cohort

Patients diagnosed with advanced stage NSCLC received treatment at the Department of Pulmonology, Semmelweis University Medical Center from November 2015 to March 2017. Each patient provided written informed consent, and necessary authorization was received from the Institutional Ethics Committee of Semmelweis University Clinical Center (#238-2/2015). There were 95 pathologically confirmed, stage IIIB-IV (advanced) NSCLC patients, and 80 NSCLC patients with concurrent COPD. The control groups consisted of 60 healthy smokers and 54 COPD patients. Performance status as defined by Eastern Cooperative Oncology Group (ECOG) (77) and clinical stage NSCLC as defined by Quint (78) were assessed. Evaluation of treatment effects on tumor, metastatic lymph nodes, or distant metastases was conducted following RECIST 1.1 guidelines. Progression-free survival (PFS) was determined as the time from the initiation of first-line therapy until disease progression or death. Patients had chest X-ray and clinical examination every 3 weeks to monitor progression. If any signs or symptoms indicated progression, CT, PET-CT, and/or MRI were conducted. At least once every three months, CT scans of the chest and upper abdomen were conducted. Overall survival (OS) was calculated from the initiation of oncological therapy to death. The diagnosis of COPD was made based on the criteria outlined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2017 (79). Our COPD patients were classified as GOLD stage II

or III. Not a single COPD patient, whether with or without cancer, experienced acute exacerbation. Treatment for all COPD patients, regardless of whether they had NSCLC or not, involved inhaled long-acting β2 agonists (LABA), long-acting muscarinic antagonists (LAMA), inhaled corticosteroids (ICS) plus LABA or ICS+LAMA+LABA. No patients or participants included in the study had utilized systemic steroids or antibiotics for a minimum of two months prior to the examination. Exclusion criteria included diagnosis of another tumor or other conditions linked to immunodeficiency or systemic inflammation. A peripheral venous blood sample was obtained upon diagnosis, prior to the initiation of any targeted or cytostatic anticancer medication. No patient had received immunotherapy at that time. A calculation was made to determine the smoking history and body mass index (BMI) of individuals. Additionally, routine laboratory parameters, as concentration of C-reactive protein (CRP) and hemoglobin (Hb), or lymphocyte (Ly) cell counts, absolute monocyte cell counts (Mo), absolute neutrophil cell counts (ANC), and platelets were assessed. Forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV1) were measured using a Total Body Plethysmograph (Piston, Budapest, Hungary) after inhaled bronchodilator.

### 3.2. Analysis of cytokines

Serum concentrations of IL-10, TNFα and IFNγ were quantified using multiplex cytometric bead-based immunoassays (Bio-Plex system from Bio-Rad in Hercules, CA, USA). VEGF concentration in serum samples was quantified using a commercially available ELISA kit (R&D Systems, Minnesota, USA). The assays were carried out in accordance with the instructions provided by manufacturers.

### 3.3. Flow cytometry

Tubes containing sodium heparin (Vacutainer, Becton Dickinson, Franklin Lakes, USA) were used to collect peripheral venous blood samples. We followed the procedures for cell preparation and flow cytometric analysis that were previously detailed (80). In a nutshell, PBMCs were extracted from the blood samples within three hours of collection using Ficoll density gradient centrifugation (Biochrom, Berlin, Germany). Immediately following the isolation of PBMCs, MDSC measures were taken. Prior to conducting the depleted T cell assays, the PBMC samples were frozen and preserved in a biobank. Prior to stimulation and flow cytometric studies, PBMCs were frozen in a solution containing 45% fetal bovine serum and 10% DMSO in complete RPMI 1640. For measuring CTLA-

4 and intracellular IFN-γ, the cells were defrosted and then cultured in RPMI 1640 medium that was supplemented with 1% MEM nonessential amino acids and vitamins (Sigma), 1 mM sodium pyruvate (Sigma), 2 mM L-glutamine (Sigma), 100 μg/ml streptomycin (Sigma), 100 μg/ml kanamycin (Gibco), 100 U/ml penicillin and 10% heat-inactivated FCS (Sigma). The cells were then stimulated with ionomycin (1 μg/mL (Sigma) and PMA (25 ng/ml; Sigma) for a period of 6 hours. Finally, the cells were stimulated with brefeldin A (10 μg/ml; Sigma) for the final two hours. Cell permeabilization and intracellular staining strategy were performed using the Cytofix/Cytoperm Fixation and Permeabilization Kit (Beckton Dickinson). Flow cytometry data were obtained using FACSAria (Beckton Dickinson) and Navios (Beckman Coulter in Brea, USA). Generated data were analysed using Kaluza software from Beckman Coulter. Flow cytometry techniques were performed using the following anti-human antibodies: phycoerythrin (PE)-conjugated anti-IFN-γ (4S.B3) and PE-conjugated anti-CD33 (WM53), PE/Dazzle 594-conjugated anti-HLA-DR (L234), PE-indotricarbocyanine (Cy7)-conjugated anti-CD14 (M5E2), allophycocyanin (APC)-conjugated anti-CD11b (ICRF44), peridinin chlorophyll protein-cyanine 5.5 (PerCP-Cy5.5)-conjugated anti-CD66b (G10F5), fluorescein isothiocyanate (FITC)-conjugated anti-CD15 (HI98) and isotype-matched control antibodies conjugated to PE/Dazzle 594, Alexa Fluor 647, PE (MOPC-21; all from BioLegend, San Diego, USA); PE/Dazzle 594-conjugated anti-CD8 (sk1), PE-Cy7-conjugated anti-CTLA-4 (CD152; L3D10), APC-Cy7-conjugated anti-PD-1 (CD279, EH12.2H7), Alexa Fluor 647-conjugated anti-Granzyme B (GB11), PerCP-Cy5.5-conjugated anti-CD3 (UCHT1), FITC-conjugated anti-CD4 (sk3); all from Sony Biotechnology, San Jose, USA); isotype-matched control antibodies conjugated to PE-Cy7, APC, APC-Cy7, PerCP-Cy5.5 and FITC (MOPC-21; all from Beckton Dickinson); APC-eFluor 780 anti-human CD3 (UCHT1), CD19 (HIB19), CD56 (CMSSB) and isotype-matched control antibodies conjugated to APC-eFluor 780 (P3.6.2.8.1; all from eBioscience, Affymetrix, Santa Clara, USA). For dead cell discrimination, eBioscience's fixable viability dye (eFluor 780, referred to as e-780 on figure) was utilized. The spontaneous cell surface expression of PD-1, CTLA-4, as well as IFN-γ production, expression were assessed in both $CD3^+CD4^+$ T helper and $CD3^+CD8^+$ T killer cells following PMA/ionomycin stimulation to evaluate the degree of

exhaustion in T cell populations. Furthermore, the intracellular granzyme-B concentration was quantified in $CD3^+CD8^+$ T killer cells.

The MDSC populations were classified as granulocytoid-(G-)MDSC: $Lin^-HLA\text{-}DR^-CD11b^{high}CD15^{high}CD33^{low}CD66b^+$ cells and monocytoid-(M-)MDSC: $CD3^-CD19^-CD56^-CD14^+HLA\text{-}DR^-CD11b^+CD33^{high}$ (Figure 1), as defined by Bronte et al (81). Lineage markers (Lin) included CD3, CD14, CD19 and CD56. Following density gradient centrifugation, peripheral blood samples, particularly in cancer patients, were shown to include low-density granulocytes in addition to mononuclear cells. G-MDSCs were quantified as a percentage of all white blood cells in samples following density gradient centrifugation, which included also peripheral blood mononuclear cells (PBMCs) and low-density granulocytes. Hence, genuine peripheral blood mononuclear cells (lymphocytes and monocytes) were recognized based on their size and complexity on the FSC-SSC plot. The M-MDSCs were then quantified as a percentage of the gated PBMCs.

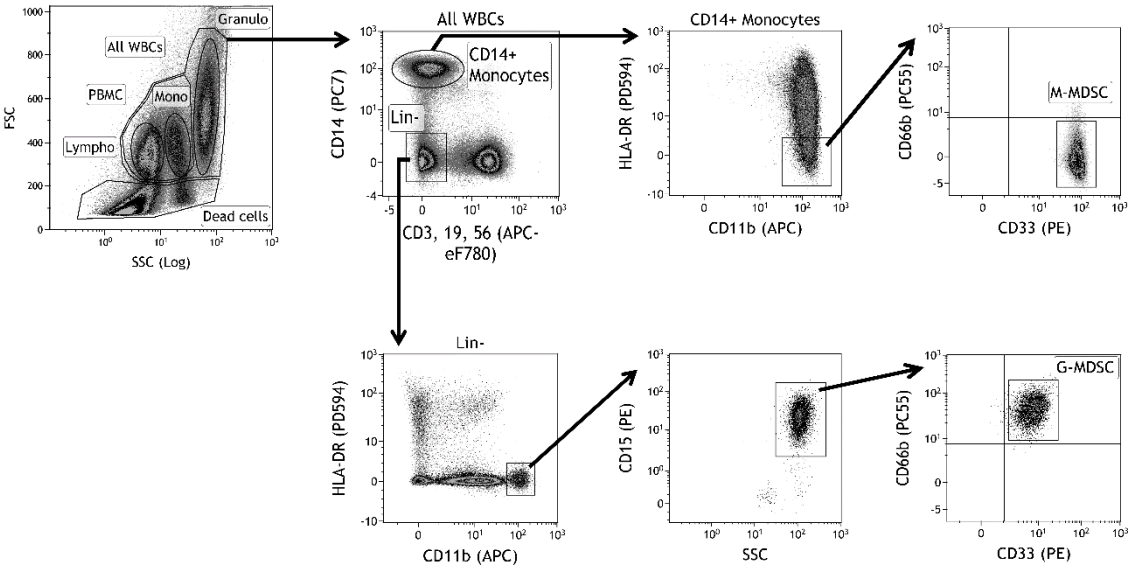


Figure 1. Flow cytometric identification of myeloid-derived suppressor cells (MDSCs). Adopted from (82) under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

### 3.4. Immunohistochemical detection and evaluation of immune cell types

Monoclonal antibodies against NKp46 (R&D Systems, Abingdon, United Kingdom) and FoxP3 (236A/E7; eBioscience, San Diego, California, USA) were utilized in order to

perform immunohistochemical staining on tissue sections of formalin-fixed, paraffin-embedded (FFPE) tumor samples. To inhibit endogenous peroxidases, deparaffinized sections were treated with 3% hydrogen peroxide ($H_2O_2$) in methanol. Antigen retrieval was then carried out by heating the sections at 98 $^oC$ in citrate buffer (pH 6.0) for 40 minutes. Then, the sections were incubated with a protein blocking solution (Protein Block, Serum-Free, Dako, Glostrup, Denmark) for 10 minutes at room temperature. Finally, the sections were incubated overnight at 4 $^oC$ with the primary antibodies. The staining was detected using the SS$^{TM}$ One-Step Polymer-HRP IHC Detection System (BioGenex, Fremont, CA, USA). The chromogene for the staining was 3-amino-9-ethylcarbazole (BioGenex), and hematoxylin counterstaining was used. The density of NKp46+ and FoxP3+ lymphocytes was evaluated following the previously established method (83). In a nutshell, we counted the number of labeled cells in 5 areas with the highest number of positive cells (hotspots), using a grid of 10x10 squares to represent an area of 0.0625 mm2 at 400x magnification. Two researchers (A.L. and M.S.) conducted independent evaluations blinded to the clinical information. The analysis was based on the mean value of their individual cell counts. The median cell density values were used as cutoff levels in analyses comparing samples with high and low immune cell infiltration level.

### 3.5. Analysis of KRAS mutation status (RFMD - Restriction Fragment Microfluidic based Detection)

Genomic DNA was isolated from FFPE tissue using the Roche High Pure PCR Template Preparation Kit (Basel, Switzerland) according to the manufacturer's instructions. DNA amplifications were performed using AmpliTaq Gold DNA polymerase from Thermo Fisher Scientific (Waltham, MA, USA), equipped with the Mastercycler gradient heat cycler from Eppendorf (Hamburg, Germany). The reactions comprised 200 µM of each dNTP, 2.5 µl 10× PCR buffer + $Mg^{2+}$, 1 pM of each primer, and 0.8 U of AmpliTaq Gold DNA polymerase. In the PCR product, which was produced using a mismatch primer as the sense primer, the BstNI or BglI restriction endonuclease recognition site of the wild-type KRAS gene was detected. Primary primer pairs have been found as codon 12 5'-GGTCCTGCACCAGTAATATG-3' and 5'-GAATATAAACTTGTGGTAGTTGGACCT-3', codon 13 5'-GGTCCTGCACCAGTAATATG-3' and 5'-

GAATATAAACTTGTGGTAGTTGGACCT-3'. Both reactions consisted of 38 cycles: one minute during denaturation at 95 ºC, one minute during primer annealing at 55 ºC, and two minutes during chain elongation at 72 ºC. The amplicon products were digested using 80 U BstNI (New England BioLabs, Ipswitch, MA, USA) at codon 12 and 80 U BglI at codon 13. For codon 12 and codon 13, four hours of enzymatic digestions in a 30 µL total volume were conducted at 60 ºC and 37 ºC, respectively. Digested PCR data were evaluated using a microfluidic-based Experion gel electrophoresis apparatus (Experion TM DNA 1K Analysis Kit, Bio-Rad Laboratories, Hercules, CA, USA). The sensitivity threshold, which was determined by the density ratio of the alternative band to the wild-type band, classified samples with more than 5% of the alternative band as mutation positive.

### 3.6. Statistics

Categorical data were compared using the Pearson $\chi^2$ test (or Fisher exact test if necessary). The normality test was used to assess continuous data. In the presence of a normal distribution, continuous variables were displayed as the mean ± standard error of the mean (SEM). When a normal distribution was not present, the median and {interquartile range} were used. For continuous data, we utilized Student's t-test to compare two groups when distribution was normal; for asymmetrical distributions, we used the Mann-Whitney U test. For comparisons of more than two groups, continuous data with a normal distribution were analyzed using one-way ANOVA and Pearson test. If the distribution was asymmetrical, the Kruskal-Wallis test and Spearman test were used. For one-way ANOVA, we utilized Tukey's post hoc test, and for Kruskal-Wallis, we utilized Dunn's test. Statistical significance was established at $p<0.05$, and the p-values are inherently two-sided. In order to compare results, we utilized the log-rank test and the Kaplan-Meier method to look for differences in OS and PFS between the groups. The independent prognostic factors for OS and PFS were assessed using Cox proportional hazards regression analysis. In addition to the immune cell density values, the model in the early stage pulmonary adenocarcinoma cohort included clinicopathological parameters that might have affected PFS or OS. These parameters included gender (female / male), age (continuous variable), smoking status (non-smokers / smokers), pathological stage (ordinal variable - stage IA-IIIA), KRAS status (wild type / mutant) and adjuvant chemotherapy (no / yes). The model in the advanced stage non-small cell

lung carcinoma cohort incorporated clinicopathological parameters that might have influenced PFS or OS. These parameters include age (older or younger than 65 years), gender (female / male), chronic obstructive pulmonary disease (COPD) status (no / yes), ECOG performance state (0 / 1-2), histology (adenocarcinoma / squamous cell carcinoma), clinical stage (IIIB / IV), adjuvant chemotherapy (yes / no), radiotherapy (yes / no), CRP (low / high), ANC (low / high) and Neu/Ly ratio (low / high). The statistical analysis was carried out using GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, CA, USA) and IBM SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA).

# 4. Results

## 4.1. Early-stage pulmonary adenocarcinoma cohort

### 4.1.1 Clinical data

Our study comprised 115 individuals with stage I-IIIA lung adenocarcinoma who underwent surgical resection. A summary of their clinical data can be found in Table I.

Table I. Clinical data of early stage surgically treated patients with lung adenocarcinoma

| Variable | Number of patients |
|---|---|
| Gender | |
| Female | 73 |
| Male | 42 |
| Age (years) | 60.3±0.9 |
| Smoking status | |
| Never smokers | 52 |
| Smokers (pack-year) | 63 (38.1±2.2) |
| Pathologic stage | |
| IA | 33 |
| IB | 51 |
| IIA | 8 |
| IIB | 10 |
| IIIA | 13 |
| KRAS mutational status | |
| Wild-type | 95 |
| Mutant | 20 |
| Adjuvant chemotherapy | |
| Yes | 72 |
| No | 43 |

Adopted from (84).

63 patients had a smoking history, with an average pack-year of 38.1±2.2, and 63% of the patients were female. The mean age was 60.3±0.9 years. Driver mutations in KRAS gene codons 12 and 13 were found in 17% of cancers. Three-quarters of the tumors was in pathological stage I. The percentage of patients who had undergone adjuvant chemotherapy was 63%.

### 4.1.2. Intratumoral NK-cell and Treg-cell density

We wondered whether the immune cells identified by immunohistochemistry were situated in the tumor's stroma or within the tumor itself. The tumor stroma was found to be the primary accumulation site for both $FoxP3^+$ and $NKp46^+$ cells, according to immunohistochemistry (Figure 2). Comparing $FoxP3^+$ and $NKp46^+$ cells in hotspots, the median densities were 1035 {744-1256} and 95.2 {62.4-132.8} cells/$mm^2$, respectively.

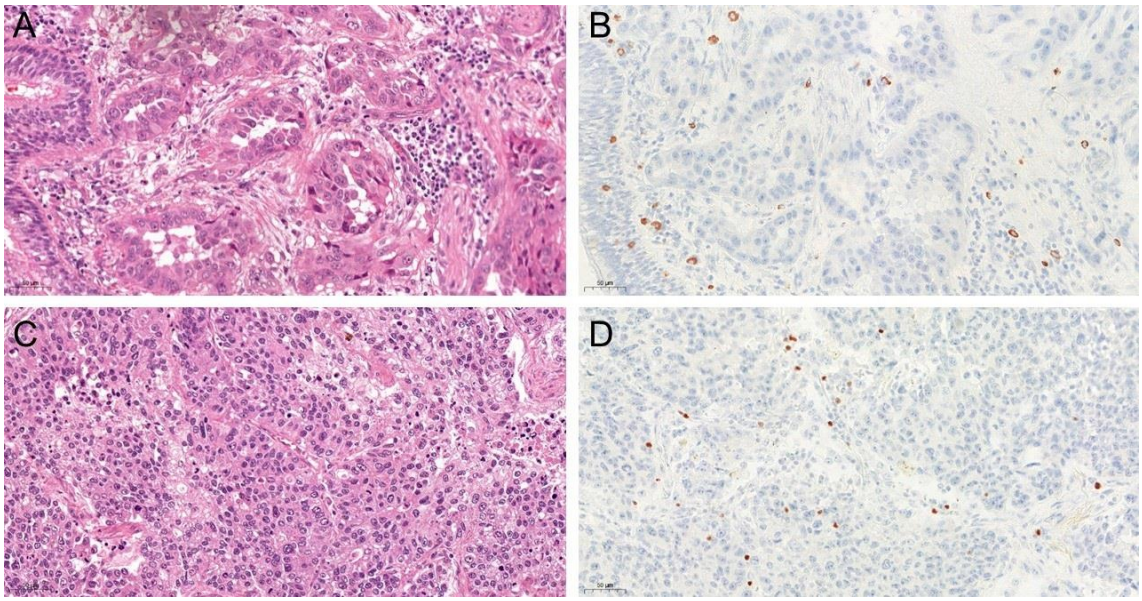


Figure 2. Hematoxylin-eosin staining of pathological stage IB adenocarcinoma of a 65 years old female patient (A) and immunohistochemical staining of $NKp46^+$ lymphocytes (B). Hematoxylin-eosin staining of pathological stage IB adenocarcinoma of a 53 years old male patient (C) and immunohistochemical staining of $FoxP3^+$ lymphocytes (D) (3-amino-ethylcarbazole: red, scale bar: 50 μm). Adopted from (84).

### 4.1.3. Differences in NK-cell and Treg-cell density according to gender, smoking status, KRAS mutation status and other clinicopathological factors

Utilizing the Mann-Whitney test, the analysis revealed that KRAS wild-type patients ($p=0.0137$), females ($p=0.0311$) and non-smokers ($p=0.0485$) had a higher number of NK cells in their tumor tissue compared to KRAS mutant patients, men, and smokers, respectively (Figure 3). In addition, patients were categorized into subgroups according to the median density of each kind of immune cell. The $\chi^2$ test was used to compare clinicopathological features is subgroups with high and low densities of immune cells.

DOI:10.14753/SE.2025.3227

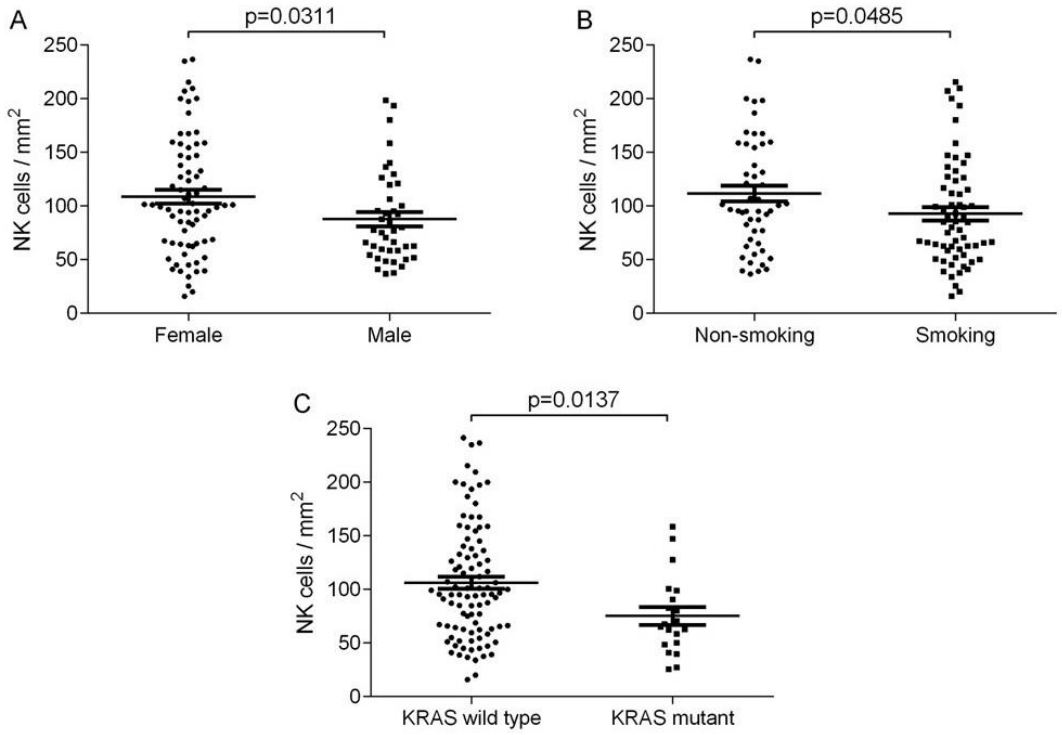


Figure 3. NK cell density in the tumor tissue of female vs. male (A), non-smoking vs. smoking (B), and KRAS wild type vs. mutant patients (C). Adopted from (84).

A statistically not significant trend was also shown in the second analysis between smoking status and NK cell density, but there was a significant correlation between NK cell density and gender or KRAS mutation status, with a higher incidence of women and KRAS wild type cases in the high NK group (Table II). Nevertheless, there was no association discovered among NK-cell density and age, pathological stage, or adjuvant chemotherapy. Moreover, no association was discovered between any of the clinicopathological factors analyzed and the density of Treg cells (Table II).

Table II. Clinical data of surgically treated patients with adenocarcinoma according to NK-cell or Treg-cell density

| Variable | Low NK group (n=57) | High NK group (n=58) | p value | Low Treg group (n=57) | High Treg group (n=58) | p value |
|---|---|---|---|---|---|---|
| Gender | | | | | | |
| Female | 31 | 42 | | 34 | 39 | |
| Male | 26 | 16 | **0.0447**# | 23 | 19 | 0.3979# |
| Age (years) | 59.8±1.4 | 60.8±1.2 | 0.581* | 60.3±1.3 | 60.3±1.3 | 0.987* |
| Smoking status | | | | | | |
| Never smokers | 21 | 31 | | 24 | 28 | |
| Smokers | 36 | 27 | 0.0736# | 33 | 30 | 0.5062# |
| Pack-year of smokers | 36.5±3.1 | 40.2±3.4 | 0.431* | 39.1±3.4 | 37.0±3.0 | 0.659* |
| Pathological stage | | | | | | |
| IA | 14 | 19 | | 16 | 17 | |
| IB | 27 | 24 | | 26 | 25 | |
| IIA | 2 | 6 | | 4 | 4 | |
| IIB | 4 | 6 | | 3 | 7 | |
| IIIA | 10 | 3 | 0.1309# | 8 | 5 | 0.6746# |
| KRAS status | | | | | | |
| Wild-type | 43 | 52 | | 47 | 48 | |
| Mutant | 14 | 6 | **0.0443**# | 10 | 10 | 0.9659# |
| Adjuvant chemotherapy | | | | | | |
| Yes | 35 | 37 | | 33 | 39 | |
| No | 22 | 21 | 0.7912# | 24 | 19 | 0.3003# |

#: $\chi^2$ test, *: t-test, statistically significant results indicated with **bold** font. Adopted from (84).

### 4.1.4. NK-cell and Treg-cell densities influence progression-free survival and overall survival

We investigated potential associations between NK and Treg cell infiltration and PFS or OS to assess their prognostic significance. Based on Kaplan-Meier analysis, patients with NK or Treg cell densities above the median had a longer PFS compared to those with densities below the median (p=0.0293 and p=0.0375, respectively). Likewise, patients

DOI:10.14753/SE.2025.3227

with a high density of NK or Treg cells in the tumor tissue were associated with a longer OS (p=0.0310 and p=0.0448, respectively, Figure 4. A-D). There was no association between the densities of NK and Treg cells, which suggested that distinct regulatory mechanisms may have been affecting the two lymphocyte subsets in pulmonary adenocarcinoma patients.

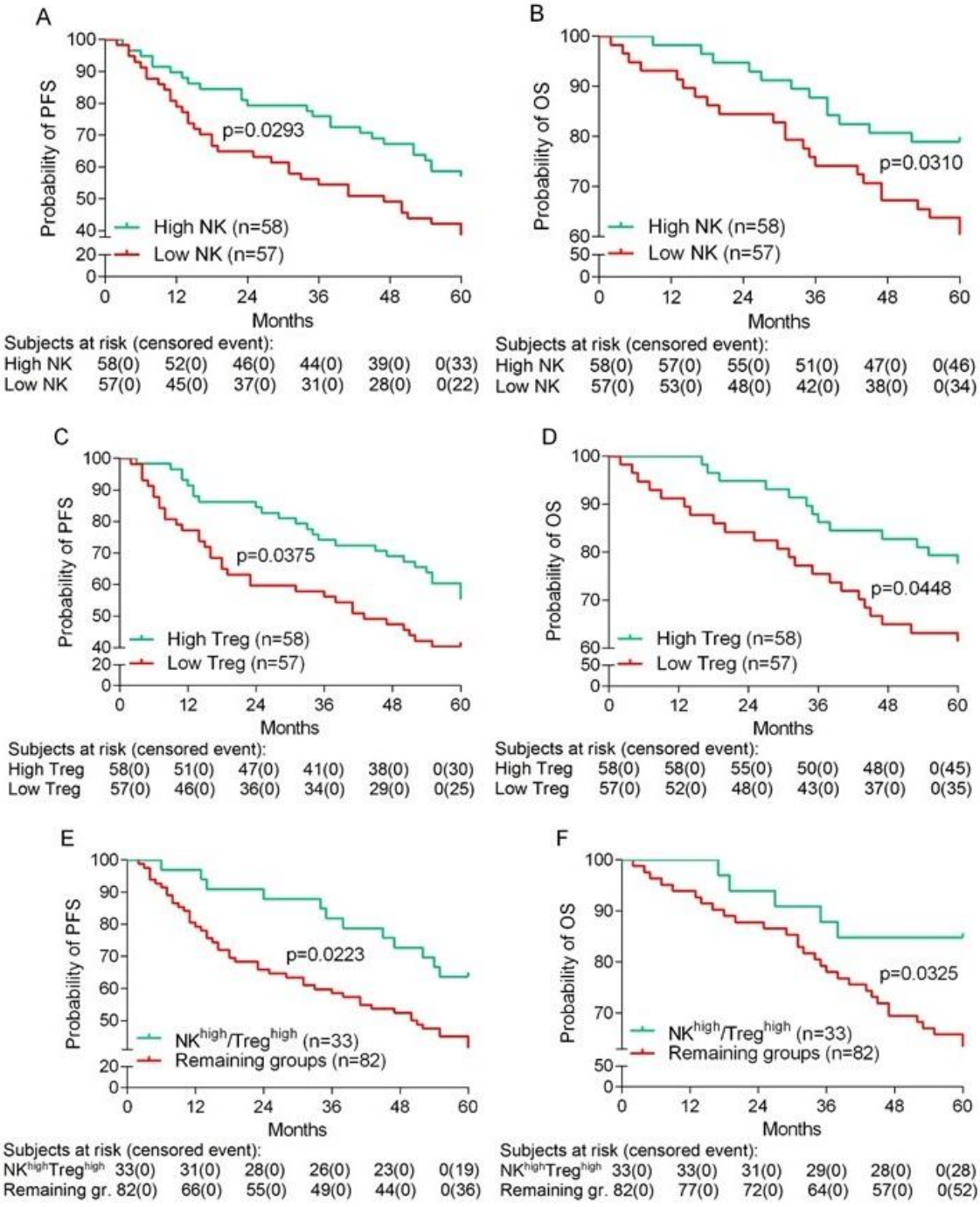

Figure 4. Progression-free (A, C, E) and overall survival (B, D, F) of patients according to NK (A, B) or Treg cell (C, D) density, or patients with combined $NK^{high}/Treg^{high}$ densities compared to Remaining groups (E, F). Adopted from (84).

An assessment of the prognostic impact of the *combination* of NK and Treg cell densities showed that high levels of NK and Treg cells prognosticated significantly longer PFS and OS compared to remaining groups (p=0.0223, respectively) (Figure 4. E, F). Remaining groups consisted $NK^{low}Treg^{high}$ group, $NK^{high}Treg^{low}$ group and $NK^{low}Treg^{low}$ group. When comparing patients whose tumors had low amounts of both cell types ($NK^{low}Treg^{low}$ group) to those whose tumors had high densities of only one cell type ($NK^{low}Treg^{high}$ group or $NK^{high}Treg^{low}$ group), there was no statistically significant difference in PFS or OS.

### 4.1.5. Multivariant Cox regression analysis of progression-free and overall survival

Additional variables that could influence PFS or OS were also examined using Cox regression analysis. These variables included adjuvant chemotherapy, age, gender, KRAS mutation status, pathological stage and smoking status (Tables III and Table IV).

Table III. Multivariant Cox proportional hazards regression analysis of PFS

| PFS<br>Variable | Analysis 1<br>HR (95% CI) | p value | Analysis 2<br>HR (95% CI) | p value |
|---|---|---|---|---|
| Age | 1.041(1.011-1.072) | **0.007** | 1.044 (1.014-1.076) | **0.004** |
| Adjuvant chemoth. (no / yes) | 1.824 (0.996-3.343) | 0.052 | 1.809 (0.993-3.294) | 0.053 |
| Gender (female / male) | 0.699 (0.378-1.294) | 0.254 | 0.849 (0.474-1.522) | 0.582 |
| KRAS status (wild-type /mutant) | 1.093 (0.520-2.297) | 0.815 | 1.217 (0.595-2.489) | 0.591 |
| Smoking (non-smokers / smokers) | 0.875 (0.509-1.504) | 0.630 | 0.985 (0.576-1.684) | 0.957 |
| Pathological stage | 1.625 (1.348-1.958) | **<0.001** | 1.651 (1.368-1.993) | **<0.001** |
| NK ($NK^{low}$ / $NK^{high}$) | 0.513 (0.285-0.925) | **0.029** | - | |
| Treg ($Treg^{low}/Treg^{high}$) | 0.621 (0.364-1.059) | 0.080 | - | |
| NK / Treg (Remaining groups / $NK^{high}Treg^{high}$) | - | | 0.474 (0.247-0.913) | **0.026** |

*Statistically significant results indicated with **bold** font. Adopted from (84).

Independent prognostic factors of PFS were older age and advanced pathological stage, as revealed by multivariate analysis. Additionally, higher than median NK cell density was identified as an independent favorable prognostic factor in these patients. Treg cell density did not achieve statistical significance in the multivariate analysis of PFS (p=0.080). Nevertheless, Treg cell density – along with age, stage, and NK cell density – was an independent prognostic factor of OS. A further analysis using the same clinicopathological data showed that the $NK^{high}/Treg^{high}$ combination was also an independent prognostic factor for both PFS and OS. (Tables III, Table IV).

Table IV. Multivariant Cox proportional hazards regression analysis OS

| OS<br>Variable | Analysis 1<br>HR (95% CI) | p<br>value | Analysis 2<br>HR (95% CI) | p<br>value |
|---|---|---|---|---|
| Age | 1.068 (1.025-1.113) | **0.002** | 1.071 (1.028-1.116) | **0.001** |
| Adjuvant chemoth. (no / yes) | 0.859 (0.390-1.893) | 0.706 | 0.845 (0.391-1.825) | 0.667 |
| Gender (female / male) | 1.126 (0.522-2.428) | 0.762 | 1.382 (0.664-2.877) | 0.387 |
| KRAS status (wild-type / mutant) | 1.529 (0.582-4.020) | 0.389 | 1.489 (0.560-3.657) | 0.399 |
| Smoking (non-smokers / smokers) | 0.809 (0.392-1.667) | 0.565 | 0.931 (0.455-1.905) | 0.844 |
| Pathological stage | 1.741 (1.374-2.207) | **<0.001** | 1.783 (1.405-2.263) | **<0.001** |
| NK ($NK^{low}$ / $NK^{high}$) | 0.534 (0.243-1.171) | **0.047** | - | |
| Treg ($Treg^{low}/Treg^{high}$) | 0.492 (0.238-1.019) | **0.046** | - | |
| NK / Treg (Remaining groups / $NK^{high}Treg^{high}$) | - | | 0.336 (0.126-0.893) | **0.029** |

*Statistically significant results indicated with **bold** font. Adopted from (84).

## 4.2. Advanced stage non-small cell lung cancer cohort

### 4.2.1. Clinical data

There was no difference between the NSCLC+COPD, NSCLC és COPD groups according to gender, age and smoking history. The majority of NSCLC patients, whether they had COPD or not, were in clinical stage IV (Table V). Adenocarcinoma predominated over squamous cell carcinoma in both NSCLC groups; nevertheless, the ratio of different histologic types was comparable in both groups. In the COPD and the NSCLC+COPD groups the $FEV_1$/FVC ratio was lower than 0.7, and $FEV_1$ was less than

80% of predicted. On the other hand, BMI was lower in the NSCLC and NSCLC+COPD groups. Patients received platinum based combined chemotherapy. In adenocarcinoma patients, standard treatment was platinum + paclitaxel + bevacizumab. In both the NSCLC and the NSCLC+COPD groups, the proportion of different therapeutic modalities, such as radiotherapy, was comparable.

Table V. Clinical data, routine laboratory parameters, PFS and OS

| Variable | Control n=60 | COPD n=54 | NSCLC n=95 | NSCLC+COPD n=80 |
|---|---|---|---|---|
| Male | 15 | 27 | 54 | 50 |
| Female | 45 | 27 | 41 | 30 |
| Age (years) | 54.3±0.9 | **63.2±1.0**$^{***}$ | **63.3±0.9**$^{***}$ | **65.3±0.9**$^{***}$ |
| Smoking (pack-year) | 28 {15-45} | **42{31-50}**$^{***}$ | 40{28-48} | **40{30-50}**$^{*}$ |
| Oncological stage | | | | |
| IIIB | - | - | 12 | 14 |
| IV | - | - | 83 | 66 |
| Adenocarcinoma | - | - | 73 | 60 |
| Squamous cell cc. | - | - | 22 | 20 |
| ECOG state | | | | |
| 0 | - | - | 56 | 45 |
| 1 | - | - | 35 | 32 |
| 2 | - | - | 4 | 3 |
| $FEV_1$ (% of predicted) | 103.1±2 | **46.1±2**$^{***}$ | **78.8±2**$^{***\#\#\#}$ | **62.0±2**$^{***\#\#\#+++}$ |
| $FEV_1$/FVC | 81.7±1 | **51.1±2**$^{***\#\#\#}$ | **79.2±2**$^{\#\#\#}$ | **62.5±2**$^{***\#\#\#+++}$ |
| BMI ($kg/m^2$) | 26.9±1.2 | 28.3±1.1 | **26.2±0.8**$^{\#}$ | **24.2±0.8**$^{\#}$ |
| Treatment | | | | |
| Platinum+gemcitabine | - | - | 14 | 11 |
| Platinum+pemetrexed | - | - | 15 | 13 |
| Platinum+taxan | - | - | 14 | 9 |

| | | | | |
|---|---|---|---|---|
| Platinum+taxan+bevac. | - | - | 48 | 40 |
| Other ¤ | - | - | 4 | 7 |
| Radiotherapy | - | - | 32 | 20 |
| No radiotherapy | - | - | 63 | 60 |
| WBC (G/L) | 7.2±0.2 | **9.0±0.3*** | **11.9±0.5**$^{***\#\#\#}$ | **10.3±0.4**$^{***+}$ |
| ANC (G/L) | 4.3±0.2 | 5.7±0.3 | **9.2±0.4**$^{***\#\#\#}$ | **7.5±0.3**$^{***\#\#++}$ |
| Ly count (G/L) | 2.3±0.1 | 2.4±0.1 | **1.7±0.1**$^{***\#\#\#}$ | **1.9±0.1**$^{\#\#}$ |
| Neu / Ly ratio | 1.9±0.1 | 2.7±0.2 | **7.41±0.8**$^{***\#\#\#}$ | **4.5±0.3**$^{**+++}$ |
| Monocytes (G/L) | 0.42±0.02 | 0.56±0.03 | **0.62±0.03**$^{***}$ | **0.67±0.04**$^{***}$ |
| Hemoglobin (g/L) | 143±2 | 148±2 | **133±2**$^{**\#\#\#}$ | **137±2**$^{\#\#}$ |
| Platelets (G/L) | 240±7 | 257±10 | **346±17**$^{***\#\#\#}$ | **324±12**$^{***\#\#}$ |
| CRP (mg/L) | 3{2-5} | 4{3-8} | **17{6-59}**$^{***\#\#\#}$ | **8{4-19}**$^{+++}$ |
| Median PFS (month) | - | - | 4.9 | **7.4**$^{++}$ |
| Median OS (month) | - | - | 11.0 | 16.9 |

Unindexed data indicate p>0.05 vs. other groups, *: p<0.05, **: p<0.01, ***: p<0.001 vs. Control, $^{\#}$: p<0.05, $^{\#\#}$: p<0.01, $^{\#\#\#}$: p<0.001 vs. COPD, $^{+}$: p<0.05, $^{++}$: p<0.01, $^{+++}$: p<0.001 vs. NSCLC; $^{¤}$other treatments: gemcitabine (n=4), docetaxel (n=3), platinum+vinorelbin (n=2), platinum+etoposid (n=2). Statistically significant results indicated with **bold** font. 

### 4.2.2. Routine laboratory data

In comparison to the control groups (Control and COPD patients), ANC was higher in the NSCLC group; however, concomitant COPD moderated it in NSCLC+COPD patients. Ly was decreased in NSCLC patients compared to Control and in NSCLC+COPD patients compared to COPD patients. The Neu/Ly ratio was drastically elevated, nearly quadrupling in NSCLC patients compared to Control, but almost halved in NSCLC+COPD patients. Elevated monocyte and platelet counts, along with reduced hemoglobin concentration, were also indicative of NSCLC and were unaffected by concurrent COPD. The CRP concentration was significantly increased, more than

fivefold in NSCLC patients compared to healthy smokers, but it was halved in NSCLC+COPD patients. Thus, concomitant COPD in NSCLC patients decreased the tumor-induced inflammation, as measured by ANC, Neu/Ly ratio and CRP concentration (Table V).

### 4.2.3. Progression-free and overall survival

In advanced malignancies, particularly NSCLC, tumor-induced inflammation is a powerful negative predictor of survival (53, 54). In line with these findings, in our cohort of patients with lower than median levels of CRP or ANC or Neu/Ly ratio has prolonged PFS. Consistent with the findings of other authors, this study demonstrated that PFS was extended in patients with mitigated inflammation as observed in NSCLC+COPD patients compared to those with NSCLC alone. The median PFS was 7.4 months for NSCLC with concomitant COPD compared to 4.9 months for NSCLC alone (p=0.0017, Figure 5.).

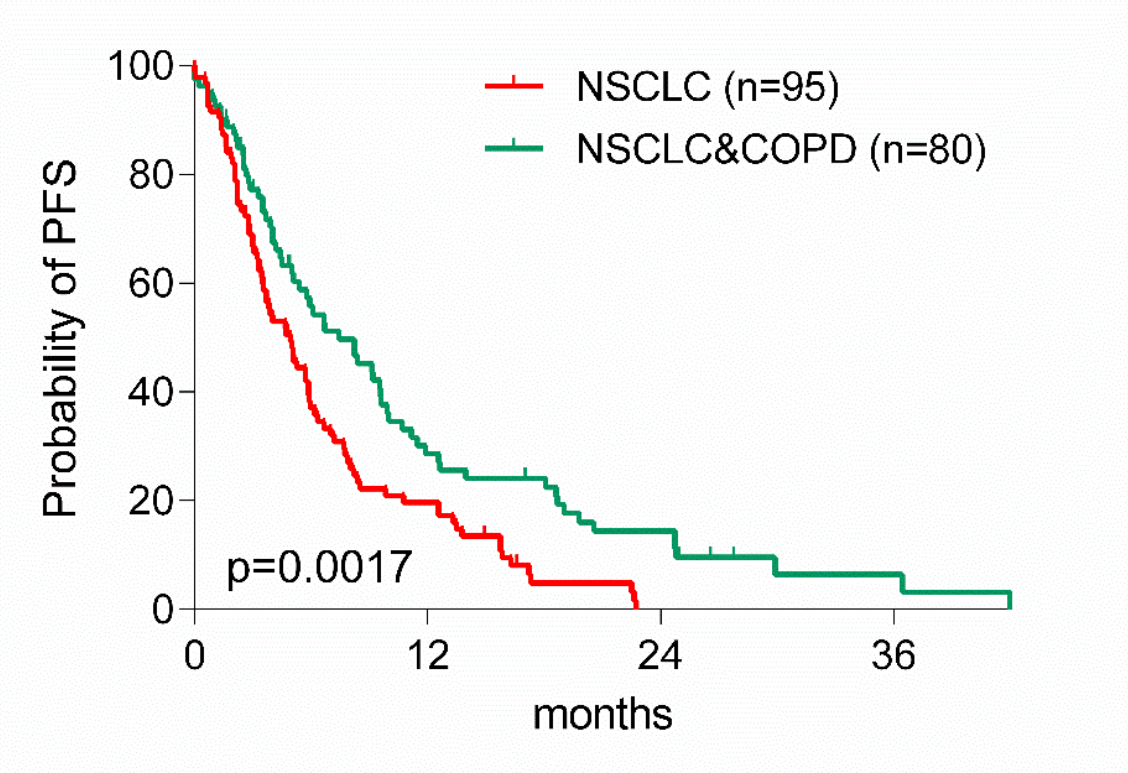


Figure 5. Kaplan-Meier analysis of progression-free survival in NSCLC and NSCLC+COPD patients. Adopted from (82) under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

The OS was numerically prolonged (16.9 months) in the NSCLC+COPD group compared to 11.0 months in NSCLC group (ns, Table V.). The hazard ratio of prolonged PFS in NSCLC+COPD patients compared to NSCLC patients was 1.7 (CI: 1.2-2.5, p=0.003, Table VI.), according to Cox proportional hazards regression analysis. Concurrent COPD, lower than median levels of CRP or ANC were identified as an independent positive prognostic factors for extended PFS after first-line treatment of advanced NSCLC.

Table VI. Cox-regression analysis of factors influencing PFS (n=175)

| Variable | | Univariate analysis | | Multivariate analysis* | |
|---|---|---|---|---|---|
| | n (%) | Median (mo) | p value | HR (95% CI) | p value |
| Age | | | | | |
| <65 years | 93 (53) | 5.7 | ns | 1.1 (0.8-1.6) | 0.575 |
| ≥65 years | 82 (47) | 5.3 | - | - | - |
| Gender | | | | | |
| Male | 104 (59) | 5.3 | ns | 1.3 (0.9-1.9) | 0.187 |
| Female | 71 (41) | 5.8 | - | - | - |
| COPD | | | | | |
| No | 95 (54) | 4.9 | **0.0017** | 1.7 (1.2-2.5) | **0.003** |
| Yes | 80 (46) | 7.4 | - | - | - |
| ECOG | | | | | |
| 0 | 101 (58) | 6.1 | - | - | - |
| 1 | 74 (42) | 4.4 | ns | 1.3 (0.9–1.9) | 0.123 |
| Histology | | | | | |
| Adenocarcinoma | 133 (76) | 5.8 | ns | 0.7 (0.4–1.1) | 0.113 |
| Squamous cell cc. | 42 (24) | 5.7 | - | - | - |
| Stage | | | | | |
| III/B | 26 (15) | 11.5 | **0.0422** | 1.4 (0.8-2.5) | 0.237 |
| IV | 149 (85) | 5.3 | - | - | - |
| Radiotherapy | | | | | |
| Yes | 52 (30) | 5.3 | - | - | - |
| No | 123 (70) | 5.9 | ns | 1.0 (0.7-1.5) | 0.948 |
| CRP | | | | | |
| Low CRP group | 88 (50) | 7.4 | **0.0426** | 1.5 (1.1-2.2) | **0.026** |
| High CRP group | 87 (50) | 4.2 | - | - | - |
| ANC | | | | | |
| Low ANC group | 88 (50) | 6.7 | **0.0141** | 1.7 (1.1-2.7) | **0.025** |
| High ANC group | 87 (50) | 5.0 | - | - | - |
| Neu/Ly ratio | | | | | |
| Low Neu/Ly ratio | 88 (50) | 6.0 | **0.0291** | 0.9 (0.6-1.4) | 0.661 |
| High Neu/Ly ratio | 87 (50) | 5.1 | - | | |

*Cox-regression model also included the type of chemotherapy, which did not influence PFS (not shown). Statistically significant results indicated with **bold** font. Adopted from (82) under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

### 4.2.4. Cytokines

To elucidate the mechanism by which simultaneous COPD moderates tumor-induced inflammation, distinct cellular and humoral components known to mediate this pathological process were examined in subgroups of each of the four study groups. The levels of proinflammatory cytokines, such as TNFα and IFNγ were significantly elevated (more than six times on average) in NSCLC, but diminished in patients with concurrent COPD. IL-10, the cytokine promoting immunologic tolerance in cancer, was more than tripled in NSCLC but halved in NSCLC+COPD (Table VII).

Table VII. Cytokines, vascular endothelial growth factor, myeloid-derived suppressor cells and T cell subpopulations

| Variable | Controls | COPD | NSCLC | NSCLC+COPD |
|---|---|---|---|---|
| Cytokines | n=24 | n=26 | n=29 | n=19 |
| IFNγ (pg/mL) | 10.8±1.9 | 36.6±5.9 | **83.0±19.9**$^{***\#}$ | **32.7±8.1**$^{+}$ |
| TNFα (pg/mL) | 7.6±1.2 | 21.8±3.7 | **38.5±6.3**$^{***}$ | **19.0±5.3**$^{+}$ |
| IL-10 (pg/mL) | 0.85±0.08 | 1.49±0.17 | **2.97±0.5**$^{***\#\#}$ | **1.45±0.22**$^{++}$ |
| VEGF, MDSC | n=27 | n=17 | n=17 | n=14 |
| Serum VEGF (pg/mL) | 545±43 | 526±67 | **1123±159**$^{**\#\#}$ | **1243±158**$^{***\#\#\#}$ |
| M-MDSC / $CD14^+$ (%) | 3.77±0.55 | 3.71±0.77 | **9.71±1.4**$^{***\#\#\#}$ | **7.88±0.84**$^{**\#}$ |
| G-MDSC / all WBCs (%) | 1.35±0.24 | 2.55±0.58 | **4.48±0.81**$^{***}$ | **2.27±0.45**$^{+}$ |
| T cell subpopulations | n=9 | n=11 | n=14 | n=13 |
| $CD3^+CD4^+$($x10^8$cell/mL) | 12.49±0.99 | 10.15±1.61 | 7.43±1.61 | 9.73±1.89 |
| $IFN\gamma^+$ ($x10^7$cell/mL) | 13.01±2.68 | 11.53±2.25 | 9.45±1.98 | 16.95±4.32 |
| Granz-$B^+$ ($x10^7$cell/mL) | 3.89±0.63 | 9.79±2.52 | 6.95±2.84 | 8.70±5.31 |
| $PD1^+$ ($x10^8$cell/mL) | 1.38±0.20 | 1.29±0.24 | 1.07±2.53 | 1.51±0.45 |
| $CTLA4^+$ ($x10^7$cell/mL) | 4.56±0.80 | 4.21±1.11 | 3.18±0.64 | 4.96±1.42 |

| | | | | |
|---|---|---|---|---|
| $CD3^+CD8^+$(x$10^8$cell/mL) | 4.11±0.49 | 5.34±1.05 | 3.10±0.66 | 4.77±0.93 |
| IFN$\gamma^+$ (x$10^8$cell/mL) | 1.36±0.34 | 1.52±0.42 | 1.24±0.29 | 2.06±0.50 |
| Granz-$B^+$(x$10^8$cell/mL) | 1.94±0.32 | 2.61±0.64 | 1.48±0.40 | **4.35±1.12$^+$** |
| $PD1^+$ (x$10^7$cell/mL) | 8.35±1.07 | 9.16±2.26 | 7.15±1.51 | 12.65±3.37 |
| $CTLA4^+$ (x$10^6$cell/mL) | 7.19±1.40 | 5.91±1.30 | 5.59±1.38 | 9.01±2.08 |

Unindexed data indicate p>0.05 vs. other groups, *: p<0.05, **: p<0.01, ***: p<0.001 vs. control, $^{\#}$: p<0.05, $^{\#\#}$: p<0.01, $^{\#\#\#}$: p<0.001 vs. COPD, $^{+}$: p<0.05, $^{++}$: p<0.01, $^{+++}$: p<0.001 vs. NSCLC). Statistically significant results indicated with **bold** font. Adopted from (82) under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

### 4.2.5. VEGF and MDSCs

Previous studies have demonstrated that in NSCLC the production of VEGF is elevated, and VEGF promotes the development of MDSCs (61-63, 85). VEGF and MDSCs were assessed in the 4 subgroups. Serum VEGF, along with granulocytoid-MDSCs (G-MDSCs) and monocytoid-MDSCs (M-MDSCs) was elevated in NSCLC patients (Table VII). In case of COPD associated with NSCLC, the M-MDSCs remained elevated compared to control groups, but numerically decreased (ns) compared to the NSCLC group. In contrast, the proportion of G-MDSCs was increased in the NSCLC group compared to the control group, but when COPD associated with NSCLC, it decreased to the range of the COPD control group. In addition, the possible correlation between serum VEGF levels and the counts of diverse cell types was analyzed. In NSCLC patients elevated serum VEGF exhibited a clear correlation with M-MDSCs (r=0.73, p<0.001, Figure 6A); however, in NSCLC+COPD patients, this correlation was reversed (r= -0.65, p=0.01, Figure 6B). In summary, in NSCLC patients concurrent COPD diminished G-MDSCs and altered or reversed the influence of VEGF on M-MDSCs, which are recognized for their immunosuppressive and proinflammatory roles in NSCLC (65, 86). G-MDSCs and serum VEGF did not correlate in any of the groups.

### 4.2.6. VEGF and white blood cells

Elevated VEGF levels were correlated with increased ANC in NSCLC patients (r=0.63, p=0.006, Figure 6C), but with concomitant COPD this correlation was ceased (r=0.32, p=0.27, Figure 6D). Conversely, no correlation was seen between absolute lymphocyte

cell count (Ly) and VEGF concentration in NSCLC patients (r=0.38, p=0.13, Figure 6E), however elevated VEGF levels were associated with increased absolute Ly counts in NSCLC+COPD patients (r=0.53, p<0.05, Figure 6F). Consequently, VEGF-related mechanisms of lymphocytopenia and neutrophilia were diminished by concurrent COPD.

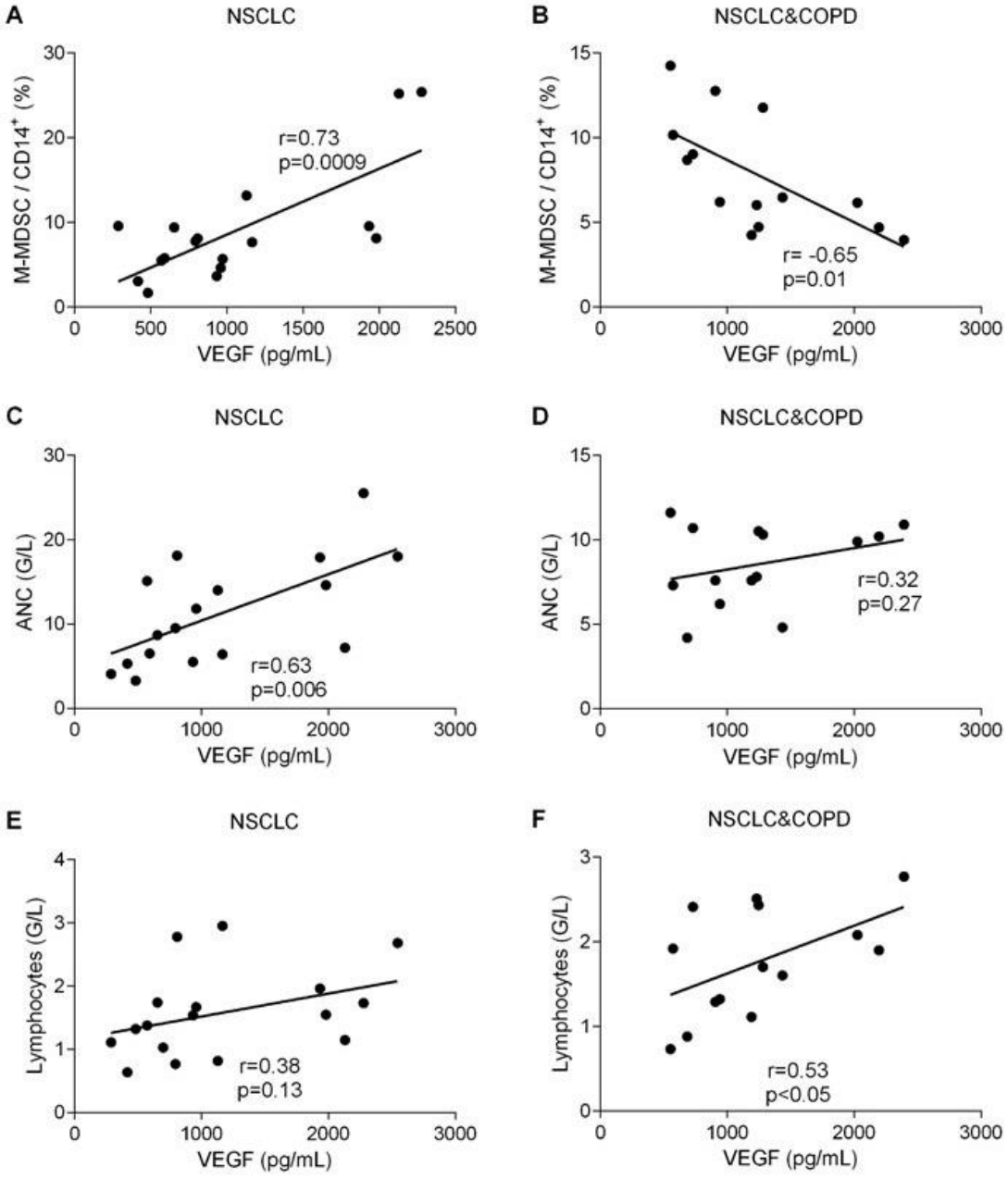


Figure 6. Relationship between serum VEGF and M-MDSC (A, B), ANC (C, D) and lymphocyte cell counts (E, F) in NSCLC (A, C, E) and NSCLC+COPD (B, D, F) patients. Adopted from (82) under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

### 4.2.7. Effector and exhausted CD4+ and CD8+ T-cells

To assess the functional status of circulating T-cells and the ratio of exhausted $CD8^+$ and $CD4^+$ T-cells, the $CD8^+$ and $CD4^+$ fractions were analyzed for intracellular granzyme-B and IFNγ contents, as well as for surface expression of the immune checkpoint molecules PD1 and CTLA4 in subsequent subgroups (Table VII). There was no evidence of systematic T cell exhaustion in either cancer group, since these lymphocyte subpopulations were of comparable size within $CD8^+$ and $CD4^+$ T-cells. Furthermore, the quantity of granzyme-B positive CD8+ T-cells was elevated in individuals with NSCLC and concomitant COPD compared to those with NSCLC alone. In addition, patients with NSCLC+COPD had reduced IL-10 levels and circulating G-MDSCs, alongside an increased presence of effector (granzim-$B^+$) $CD8^+$ T-cells. Furthermore, only patients with NSCLC and concomitant COPD exhibited a direct correlation between VEGF and absolute Ly count, while an indirect correlation was observed between VEGF and M-MDSCs, and last but not least the strong correlation between ANC and VEGF seen is NSCLC patients was absent in NSCLC+COPD patients.

### 4.2.8. Effects of concomitant COPD in bevacizumab+chemotherapy treated advanced NSCLC patients

Previous studies indicated that decreased pretreatment VEGF levels in serum (62, 63) or plasma (61, 87) are associated with prolonged survival in advanced NSCLC. Given the current findings that in NSCLC concomitant COPD modifies the relationships between MDSCs, neutrophil granulocytes, lymphocytes and VEGF, it was pertinent to investigate whether the PFS prolonging effect of concomitant COPD was preserved within patients uniformly treated with bevacizumab and chemotherapy. Consequently, two additional, otherwise comparable, stage IIIB-IV adenocarcinoma patient groups (one group without and one group with COPD) were assembled. All patients have been administered bevacizumab in conjunction with carboplatin and paclitaxel. Patients with concurrent COPD (n=35) exhibited reduced ANC, elevated absolute Ly counts, and a diminished Neu / Ly ratio (all $p<0.05$). As previously observed, PFS was shown to be prolonged in NSCLC+COPD patients ($p=0.049$) compared to NSCLC patients (n=39). Thus, anti-VEGF treatment did not neutralize the anti-inflammatory and PFS prolonging effects of concurrent COPD in NSCLC (Table VIII).

DOI:10.14753/SE.2025.3227

Table VIII. Clinical data, routine laboratory parameters, PFS and OS of stage IIIB-IV adenocarcinoma patients received bevacizumab with carboplatin+paclitaxel

| variable | NSCLC (n=39) | NSCLC+COPD (n=35) |
|---|---|---|
| Male | 20 | 17 |
| Female | 19 | 18 |
| Age (years) | 62±2 | 65±1 |
| Oncological stage | | |
| IIIB | 4 | 4 |
| IV | 35 | 31 |
| ECOG state | | |
| 0 | 27 | 23 |
| 1 | 11 | 11 |
| 2 | 1 | 1 |
| $FEV_1$ (% of pred.) | 81±3 | **62±4**$^{+++}$ |
| $FEV_1$/FVC | 73±1 | **58±2**$^{+++}$ |
| BMI (kg/m$^2$) | 24.2±0.8 | 25.3±0.9 |
| CRP (mg/L) | 15 {6-53} | **6 {3-16}**$^{+}$ |
| WBC (G/L) | 12.4±0.7 | 11.2±0.6 |
| ANC (G/L) | 10.1±0.7 | **7.8±0.5**$^{+}$ |
| Ly (G/L) | 1.6±0.1 | **2.2±0.1**$^{+}$ |
| Neu / Ly ratio | 6.7±0.7 | **4.5±0.4**$^{++}$ |
| Monocytes (G/L) | 0.6±0.0 | 0.7±0.1 |
| Hemoglobin (g/L) | 131±2 | 141±3 |
| Platelets (G/L) | 367±29 | 330±16 |
| Median PFS (months) | 3.3 | **6.1**$^{+}$ |
| Median OS (months) | 11.0 | 15.0 |

Unindexed data indicate $p>0.05$, $^{+}$: $p<0.05$, $^{++}$: $p<0.01$, $^{+++}$: $p<0.001$ vs. NSCLC. Statistically significant results indicated with **bold** font. Adopted from (82).

DOI:10.14753/SE.2025.3227

## 5. Discussion

This thesis summarizes our investigations on specific aspects of the immunological and inflammatory responses influencing the clinical course of resectable (I-IIIA) and advanced (IIIB-IV) non-small cell lung cancer. In the 1st study, we analyzed the density of natural killer cells (NK cells) and regulatory T cells (Treg cells) in tissue samples from patients with resectable lung adenocarcinoma, assessing their correlation with PFS and OS. In non-resectable (stage IIIB-IV) lung adenocarcinoma, immunotherapy (e.g. anti-PD1 or anti-PD-L1 treatment) has yielded outstanding outcomes for years, indicating that a specific immune response is activated against advanced lung cancer. Blocking the PD1 – PD-L1 interaction can restore an effective immunological connection between effector T cells and tumor cells, leading to tumor cell eradication in many patients with advanced stage NSCLC. A small subset of patients treated in this manner exhibits several years of regression; however, in a larger proportion, immunotherapy ultimately loses its effectiveness over time. The anti-tumor immune response can be disrupted by multiple mechanisms, including the accumulation of regulatory T cells and myeloid-derived suppressor cells, as well as the activation of various humoral immunosuppressive factors (VEGF, IL-10), which can impede immunological eradication of tumor.

It is apparent that relapse may occur in curatively treated stage IA-IIIA lung adenocarcinoma patients, and it occurs earlier in stage IIIA patients than in stage IA. Even with curative treatment, a sustained immune response may be crucial for the persistent elimination of residual tumor cells following curative surgery. We hypothesized that the presence of tumor-eradicating immune cells within the resected tumor might forecast (years in advance) PFS and OS. Several data indicate that a dense infiltration of T and B cells within tumors is associated with a significantly longer OS (17, 88).

NK cell accumulation within the tumor was examined together with Treg cells, which regulate the cellular immune response. Regarding the latter, we were guided by two special circumstances. One is that the oncopathological significance of Treg cell accumulation within tumors remains unclear. Innovative drug research has initiated many clinical trials based on the consideration of whether the antitumor effectiveness of Treg cell inhibition can be proven. Numerous preclinical findings suggest that inhibiting the Treg response may hold promise for clinical drug research, however, all these efforts have so far remained clinically fruitless. Another aspect is the limited number of studies

examining the simultaneous infiltration of NK and Treg cells within tumors, their relationship with the late relapse of previously resected lung adenocarcinoma.

This study confirmed the prognostic significance of NK cell density in tumoral stroma. Additionally, male patients and smokers exhibited reduced tumor-infiltrating NK cells compared to female patients and non-smokers, respectively. Jaillon et al. (89) have conducted a review on sexual dimorphism in innate immunity. They have determined that females exhibit reduced immunological susceptibility to microbial infections as well as malignancies. Cigarette smoke has been shown to diminish the cytotoxic activity and cytokine secretion of NK cells in vitro (90). In our cohort of adenocarcinoma patients NK cell accumulation was reduced in the KRAS mutant cases compared to those with a wild-type KRAS mutation status. Transcriptome sequencing analysis utilizing data from The Cancer Genome Atlas (TCGA) indicates that the G12D point mutation of KRAS, particularly in conjunction with TP53 mutation, correlates with a reduced prevalence of NK cells (91). A comprehensive study indicated that the presence of co-mutations, such as TP53, in KRAS mutant lung adenocarcinomas can affect the quality and intensity of the antitumor immune response (92).

It is widely thought that regulatory T cells promote tumor growth in solid malignancies, including non-small cell lung cancer (93). The direct interaction of Tregs with effector immune cells, along with the secretion of inhibitory cytokines, can suppress antitumor immune responses (94). Despite this observation the effect of Treg accumulation on survival in resectable NSCLC remains unclear. Multiple reports indicate a favourable correlation between Treg cell infiltration and extended survival in NSCLC (42-44), squamous cell carcinoma (42, 50) and lung adenocarcinoma (42, 43, 95). Our current findings in resectable pulmonary adenocarcinoma supports these latter findings. A high density of intratumoral Tregs has been linked to favorable prognosis in various other tumor types, including head and neck, colorectal, ovarian cancer and estrogen receptor-negative breast cancer (37-39, 96, 97).

The inconsistent prognostic findings regarding Treg accumulation in distinct solid cancers remain inadequately elucidated. Tregs located in non-lymphoid tissues, such as bronchial or intestinal mucosa, play a significant role in regulating local inflammation (98). It has been hypothesized that Treg cells, due to their anti-inflammatory activity, may exert beneficial effects in cancers characterized by an inflammatory background.

Additionally, in line with their function in modulating immune responses through negative feedback, the presence of Tregs may accompany the immune effector cells, suggesting that their accumulation could indicate an active antitumor immune response (35, 96). This hypothesis appears to be corroborated by Koh J et al. (99), who investigated two different cohorts of NSCLC patients administered immune checkpoint inhibitors (ICI). In all cohorts, patients treated with ICIs who exhibited elevated circulating Treg counts and high plasma TGFβ levels one week post-treatment demonstrated sustained clinical benefit, along with extended progression-free survival (PFS) and overall survival (OS), in contrast to patients who did not show post-treatment Treg proliferation and elevated TGFβ levels and had shorter PFS and OS.

Consequently, to determine if NK cells and Treg cells had a unidirectional effect on survival prolongation, we investigated the prognostic impact of intratumoral $FoxP3^+$ Treg density in combination with $NKp46^+$ NK cell density and discovered that both PFS and OS were markedly extended in patients exhibiting concurrent high densities of NK and Treg cells compared to other cohorts.

Tregs are recognized for their role in modulating NK cell function through various mechanisms (19). They may, for instance, prevent NKG2D-mediated tumor cell cytotoxicity by inducing downregulation of the NK cell activating receptor NKG2D (19, 100, 101). Bergmann et al. (100) observed that IL-2-induced NK cell activation was suppressed by in vitro-generated induced Tregs (iTregs) only in the absence of tumor cells; but, in the presence of tumor target cells, iTregs enhanced the tumoricidal activity of NK cells. The findings indicate a complex regulatory mechanism of NK cells by iTregs, which may partially elucidate the favorable survival prognosis associated with high Treg density in both solid tumors and some lymphomas (102, 103). Tregs not only protect and sustain self-tolerance during antitumor immune responses but also keep NK cells in a highly responsive state by counteracting the down-regulatory effects of chronic stimulation of natural killer receptors (104).

In our second study we examined patients with stage IIIB-IV non-small cell lung cancer. Given that COPD is the predominant comorbidity associated with NSCLC, we investigated whether COPD as a comorbidity influences the efficacy of first-line platinum-based (palliative) treatment, progression-free survival and overall survival.

COPD is a chronic inflammatory disease that can promote the development of NSCLC after several years. We hypothesized that the immune response and inflammation of varying intensity associated with NSCLC are influenced by the presence of concurrent COPD, a condition characterized by chronic immunological activation, inflammation, and an anti-inflammatory response. Given that alterations in immune response and inflammation are critical to the interaction between the two diseases, we examined laboratory indicators of systemic inflammation alongside the clinical course. Additionally, we assessed plasma concentrations of the proinflammatory cytokines, such as IFNγ and TNFα, as well as immunosuppressive factors (e.g. IL-10) and VEGF in smaller patient cohorts. We additionally examined the amount of MDSCs and the exhausted $CD4^+$ and $CD8^+$ T cells in four patients cohorts (NSCLC+COPD group, NSCLC gorup, COPD control group and healthy smoker control group).

Stable COPD is a chronic inflammatory condition (59, 60, 105), and its presence may synergize with tumor-induced inflammation, potentially worsening survival outcomes. This has been previously examined in early-stage (I-IIIA) NSCLC patients who underwent surgical resection of the tumor (106, 107). In these studies, OS was found to be shorter in individuals who also suffered from COPD. The mechanisms of interference between COPD and NSCLC may differ significantly between curatively treatable and palliatively treatable NSCLC. Following curative treatment for early-stage NSCLC, the anticipated OS extends to several years, whereas after palliative treatment for advanced NSCLC, the median OS is approximately 12 months (52). Moreover, alongside several adverse effects of concurrent COPD in cancer, COPD also induces various anti-inflammatory and antioxidant pathways prior to and likely during tumor formation (106, 107), COPD also maintains various antioxidant and anti-inflammatory pathways prior to and likely during tumor formation (108-110). In patients with COPD, levels of α1-antitrypsin, α2-macroglobulin, haptoglobin, orosomucoid (108), as well as ferroxidase (coeruloplasmin), surfactant protein–D (109) and glutathione peroxidase (110) are elevated in sputum, plasma or both. In addition, CD8 cells are also more prevalent in COPD (105). Additionally, tertiary lymphoid tissues develop in the bronchial walls of COPD patients, predominantly including B-cells and immunoglobulin-secreting plasma cells (111). These anti-inflammatory and immunologic mechanisms may theoretically

moderate tumor-induced inflammation and enhance therapeutic efficacy in patients with advanced NSCLC and COPD. The issue has not been extensively studied to date.

Limited reports exist regarding the impact of concomitant COPD on the progression of late-stage NSCLC. Concomitant COPD did not negatively impact survival in any of these studies. A retrospective analysis involving 50 patients revealed that concomitant COPD did not affect the proportions of patients exhibiting partial response, stable disease, or progressing disease during first-line combination chemotherapy (71). In another study (72), OS remained unchanged in NSCLC+COPD patients compared to those with only NSCLC, despite the former being much older (by 4 years, $p<0.001$) than patients without COPD. The total patient count was modest (n=85). The overall response rate (ORR) was quantitatively higher in concomitant COPD patients compared to non-COPD NSCLC patients (38.9% vs 22.9%, $p=0.14$). Izquierdo et al (73) demonstrated comparable OS in patients with concomitant COPD and non-COPD NSCLC. However, the mean age of the former group was 70 years, compared to 66 years in the latter group ($p<0.001$). Both studies had an uneven distribution of patients with EGFR mutant adenocarcinoma (long survivor genotype), a high proportion of male patients (70–90%), and a preponderance of squamous cell carcinoma.

On the other hand, Abal Arca et al. (74) found that OS was longer in patients with COPD+NSCLC (n=396) compared to patients with NSCLC alone (n=600). This was true even though the former group was 3.5 years older, comprised mostly of men (90%) and had twice the rate of squamous cell carcinoma histology.

In our study with 175 advanced NSCLC patients, we demonstrated that concurrent COPD significantly extended OS during and after first-line therapy. Several potential underlying mechanisms were identified that elucidate this previously unrecognized effect of COPD. Patients with NSCLC and COPD exhibited a reduced Neu/Ly ratio, indicative of moderated tumor-induced inflammation and / or a less suppressed antitumor immune response (57, 58). Yao et al. (57) demonstrated that a lower pretreatment Neu/Ly ratio correlates with improved PFS in advanced NSCLC following first-line chemotherapy. Teramukai et al (54) observed that lower than the median pretreatment neutrophil granulocyte counts were associated with longer PFS and OS in advanced NSCLC. Lee et al. (112) reported that an early reduction in the Neu/Ly ratio serves as a surrogate

marker for improved OS in advanced lung adenocarcinoma patients treated with gefitinib or standard first-line chemotherapy.

The alterations in the Neu/Ly ratio in peripheral blood were examined alongside the quantification of effector (IFNγ positive) CD4 and CD8 cells, as well as the exhausted subsets of these T cells in patients with surgically resected intrahepatic cholangiocarcinoma (113). The density of tumor-infiltrating CD3 cells (TIL) was assessed. Among the 102 patients, those with a higher peripheral blood Neu/Ly ratio exhibited a lower ratio of peripheral blood IFNγ-positive cells, a higher ratio of PD1-positive T cells, and fewer tumor-infiltrating lymphocytes (TILs). Patients with a Neu/Ly ratio above the median exhibited significantly shorter survival times. The authors concluded that a higher Neu/Ly ratio correlates with diminished antitumor immunity and may serve as a biomarker for unfavorable prognosis.

T cell exhaustion has been primarily assessed within tumors among tumor-infiltrating lymphocytes (TILs). However, by monitoring the percentage of $PD1^{+}CD8^{+}$ T cells in the bloodstream, Kamphorst et al. (67) were able to track the pharmacodynamic impact of pembrolizumab (anti-PD1 antibody). Huang et al. (68) conducted a peripheral blood analysis that revealed evidence of CD8 cell reinvigoration, indicated by an increased fraction of IFNγ+CD8 cells, after pembrolizumab administration. These findings indicate that the analysis of exhausted and reinvigorated T cells can be conducted in peripheral blood, which is more accessible than analyzing tumor-infiltrating lymphocytes (TILs). Our current observation of an elevated granzyme $B^{+}CD8$ fraction in patients with NSCLC and COPD compared to those with NSCLC alone corroborates other laboratory studies, indicating a less inhibited immune response in advanced NSCLC when associated with COPD.

The current investigation found that simultaneous COPD was linked to a decreased Neu/Ly ratio, suggesting diminished tumor-induced inflammation and lymphopenia. COPD is associated with recurrent bacterial and viral infections of the airways, leading to sustained innate and adaptive immune responses (105). In COPD, the production of antinuclear antibody (ANA) may occur, indicating the activation of autoimmune mechanisms associated with this condition (111). Infectious and autoimmune stimuli linked to COPD may be pertinent in NSCLC, as the targeted activation of the adaptive

immune system is recognized to suppress the development of MDSCs (114). Moreover, COPD is linked to the release of neutrophil elastase, (NE (115)), an enzyme that has been shown to be internalized by lung adenocarcinoma cells (116) and potentially rendering NE carrying lung cancer cells more immunogenic (117). However, NE has also been demonstrated to cleave VEGF into fragments that are unable to bind to their specific cellular receptors (118). Multiple studies have indicated that elevated pretreatment levels of VEGF in plasma (61, 87) or serum (62, 63) are associated with reduced PFS and OS during chemotherapy, with or without bevacizumab, in advanced NSCLC. Consequently, the associated alterations in VEGF synthesis due to COPD may have clinical significance regarding the outcomes of NSCLC with concomitant COPD. In emphysema patients, a reduction in VEGF levels was observed in sputum (119), plasma (120) and lung tissue (121). Conversely, in the bronchitic phenotype of COPD, sputum VEGF levels have been reported to be significantly elevated (119, 120). Additionally, as previously stated, NE is capable of cleaving and inactivating VEGF (118). Our findings indicate that concomitant COPD disrupts several myeloid and lymphoid responses to VEGF. Elevated VEGF levels were correlated with increased neutrophil granulocyte cell counts and M-MDSCs in NSCLC, but not in those with concurrent COPD. Furthermore, a direct correlation between VEGF and lymphocyte cell count was identified solely in the NSCLC+COPD group. As a possible explanation VEGF has been shown to activate VEGFR2-mediated signaling in T cells (122). It is possible therefore, that NSCLC was more immunogenic in those patients who also had COPD. The moderation and modulation of both inflammation and immunosuppression by concurrent COPD may have contributed to the extended PFS during first-line therapy of advanced NSCLC.

## 6. Conclusions

- NK cells infiltrate the stroma of resected lung adenocarcinoma. Increased NK cell infiltration correlates with extended PFS and OS.
- Men, smokers, and KRAS mutant patients exhibited reduced tumor-infiltrating NK cells compared to women, non-smokers and KRAS wild-type patients.
- Treg cells also infiltrate these tumors. Increased Treg cell infiltration is interestingly also accompanied with longer PFS and OS.
- PFS and OS markedly extended when both NK and Treg cell densities were above the median. Consequently, Treg cells may constitute an inherent component of the anticancer response mediated by NK cells.
- Multivariant Cox proportional hazards regression analysis revealed that younger age, earlier pathological stage, higher than median NK cell density or combined $NK^{high}/Treg^{high}$ density were independent prognostic factors of longer PFS.
- Multivariant Cox regression analysis revealed that younger age, earlier pathological stage and higher than median NK and Treg cell density or combined $NK^{high}/Treg^{high}$ density were independent prognostic factors of longer OS.
- Concomitant COPD in stage IIIB-IV NSCLC significantly prolonged PFS during and following first-line combined chemotherapy. Multivariant Cox proportional hazards regression analysis revealed that COPD is an independent prognostic factor of longer PFS in this cohort.
- Concurrent COPD diminished NSCLC-induced inflammation, mitigated immunosuppression, and enhanced the activity of few effector populations of $CD8^+$ T cells.
- In the NSCLC (without COPD) group there were direct correlations between increased VEGF levels and ANC as well as M-MDSCs, while lymphocyte count was diminished. Concomitant COPD, however, inhibited the VEGF induced neutrophilia and the proliferation of pro-tumor M-MDSC population. Concurrent COPD also supported the maintenance of normal circulating lymphocyte levels in NSCLC patients.
- Thus, concomitant COPD can reduce tumor-induced inflammation in stage IIIB-IV NSCLC. This phenomenon may contribute to the extended PFS following during and following first-line treatment.

DOI:10.14753/SE.2025.3227

## 7. Summary

Tumor immunology research aims to determine how the immune system inhibits tumor development in some people but not others and how tumor cells and local or circulating immune cells interact.

There is no clear consensus regarding the role of NK cells and Treg cells in either promoting or inhibiting the progression of NSCLC as well as their impact on PFS and OS after 1st line combined chemotherapy. Additionally, the relationship between mutations in NSCLC and the density of immune cells remains unclear. We discovered in 115 patiens with early stage adenocarcinoma that males, smokers, and interestingly, KRAS mutant patients exhibited reduced intratumoral NK cell density. Elevated NK cell density and increased Treg cell density correlated with prolonged PFS and OS. This association became even more robust when patients exhibiting combined high densities of NK and Treg cells ($NK^{high}Treg^{high}$). Increased densities of NK cells, Treg cells, and combined $NK^{high}Treg^{high}$ are established as independent prognostic factors of prolonged OS and PFS. Thus, NK and Treg cells are essential elements of anti-tumor immune response and tumor-induced inflammation, and both operates most effectively when in equilibrium.

COPD is a chronic inflammatory disease that, in additon to being the most common comorbidity of NSCLC, can promote the development of NSCLC after several years, so we investigated how concomitant COPD influences PFS or OS, and how COPD-induced inflammation and immune responsiveness alter those induced by NSCLC. In our cohort of 175 patients with advanced stage NSCLC, we found that concomitant COPD prolonged PFS following 1st line combined chemotherapy. Moreover multivariant Cox regression analysis revealed that COPD is an independent prognostic factor of longer PFS in this cohort. We discovered that in NSCLC, concurrent COPD diminished tumor-induced inflammation, mitigated immunosuppression, and enhanced $GranzB^{+}CD8^{+}$ T-cell populations, thereby promoting effector lymphoid functions. Moreover, in the NSCLC+COPD group, elevated VEGF levels did not induce neutrophilia (as in the NSCLC group) but were linked to increased lymphocyte counts (unlike the NSCLC group) and also decreased the proinflammatory M-MDSCs. According to our observations the moderation and modulation of both inflammation and immunosuppression by concurrent COPD contributed to the extended PFS during and following 1st line chemotherapy of advanced NSCLC.

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DOI:10.14753/SE.2025.3227

## 10. Acknowledgements

I would like to express my sincere gratitude to my supervisor György Losonczy MD, PhD, DSc, professor emeritus of the Department of Pulmonology at Semmelweis University for his many valuable advices, guidance and tremendous help in research.

I am grateful to Gabriella Gállfy MD, PhD for introducing me pulmonary oncology as a student in the science student group.

I would like to express my gratitude to Zsolt Komlósi MD, PhD for his leadership in flow cytometry and for imparting valuable skills for the proper use of FACS.

I am grateful to József Tóvári PhD, DSc for providing laboratory conditions and valuable advices.

I am very grateful to Andrea Ladányi PhD, DSc for the tremendous help and collaboration in the fields of tumor immunology and immunohistochemistry.

I am grateful to Gábor Barna PhD to have had the opportunity to perform flow cytometry in his lab.

I am grateful to Ildikó Horváth MD, PhD, DSc for involving me in the U-BIOPRED project and for her valuable research advices.

I express my gratitude to Csaba Bödör, PhD, DSc, for his encouragement in my research progress and for his valuable research advices.

I would like to express my gratitude to all colleagues and members of the laboratories in which I have worked, particularly Katalin Derecskei and Gergő Szűcs.

Finally, I am extremely grateful to my family, especially my beloved wife and three children, for their unwavering love and support throughout and after my PhD study.

*The research studies received funding from the Hungarian National Research, Development and Innovation Office (NKFIH) 108009 to György Losonczy, NKFIH ANN 128524 to Andrea Ladányi and NKFIH K147410 to József Tóvári. The authors acknowledge financial support from the National Laboratories Excellence Program [under the National Tumor Biology Laboratory Project (NLP-17, 2022-2.1.1-NL-2022-00010] and the Hungarian Thematic Excellence Program [TKP2021-EGA-44] to Andrea Ladányi and József Tóvári.*