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DOKTORI ISKOLA

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# Assessment of inflammatory marker expression and immune phenotypes in lung cancer

**PhD thesis**


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## List of Abbreviations

| | |
|---|---|
| AC | Atypical carcinoid |
| ALK | Anaplastic lymphoma kinase |
| APC | Antigen-presenting cell |
| BRAF | B-Raf proto-oncogene, serine/ threonine kinase |
| CASP | Competitive allele-specific polymerase chain reaction |
| CD3 | Cluster of differentiation 3 |
| CD4 | Cluster of differentiation 4 |
| CD8 | Cluster of differentiation 8 |
| CD56 | Cluster of differentiation 56 |
| CD163 | Cluster of differentiation 163 |
| CHT | Chemotherapy |
| COPD | Chronic obstructive pulmonary disease |
| DNA | Deoxyribonucleic acid |
| EGFR | Epidermal growth factor receptor |
| ERBB2 | Erb-B2 receptor tyrosine kinase 2 |
| FFPE | Formalin-fixed, paraffin-embedded |
| FGFR1 | Fibroblast growth factor receptor 1 |
| GITR | Glucocorticoid-induced tumor necrosis factor receptor |
| H&E | Hematoxylin and eosin |
| HER2 | Human epidermal growth factor receptor 2 |
| HPF | High power field |
| IC | Immune cell |
| IHC | Immunohistochemistry |

| | |
|---|---|
| IL-1β | Interleukin-1 Beta |
| IL-18 | Interleukin-18 |
| KEAP1 | Kelch-like ECH-associated protein |
| Ki-67 | Kiel-67 antigen |
| KRAS | Kirsten rat sarcoma virus |
| LADC | Lung adenocarcinoma |
| LCNEC | Large cell neuroendocrine carcinoma |
| LDCT | Low-dose computer tomography |
| LNEN | Lung neuroendocrine neoplasm |
| MEK/MAPK | Mitogen-activated protein kinase |
| METex14 | Mesenchymal-epithelial transition factor exon 14 |
| NFE2L2 | Nuclear factor, erythroid 2 like 2 |
| NK | Natural killer |
| NKG2A | CD94/NK group 2 member A 4 |
| NLRP3 | NOD-like receptor family pyrin domain-containing 3 |
| NSCLC | Non-small cell lung cancer |
| NTRK1/2/3 | Neurotrophic tyrosine receptor kinase 1/2/3 |
| OS | Overall survival |
| OTP | Orthopedia homeobox protein |
| OX40 | Tumor necrosis factor receptor superfamily, member 4 (TNFRSF4) |
| PC | Principal component |
| PD-1 | Programmed cell death protein 1 |
| PD-L1 | Programmed death-ligand 1 |

| | |
|---|---|
| PRRT | Peptide receptor radionuclide therapy |
| R | Pearson correlation coefficients |
| RET | Rearranged during transfection |
| RNA | Ribonucleic acid |
| ROS1 | C-ros oncogene one receptor tyrosine kinase |
| RT-PCR | Real-time polymerase chain reaction |
| SCLC | Small cell lung cancer |
| SSA | Somatostatin analogs |
| STK11 | Serine/threonine kinase 11 |
| TC | Typical carcinoid |
| TIL | Tumor-associated lymphocyte |
| TIM | Tumor immune microenvironment |
| TIM3 | T cell immunoglobulin and mucin domain 3 |
| TMA | Tissue microarray |
| TP53 | Tumor protein 53 |
| Treg | Regulatory T cell |
| VISTA | V-domain immunoglobulin suppressor of T cell activation |
| WHO | World Health Organization |

# 1. Introduction

## 1.1. Lung cancer epidemiology

Lung cancer remains one of the most prominent malignancies worldwide and is considered the leading cause of cancer-related mortality. In Hungary, it is the most frequently diagnosed cancer among men and the third most common among women, accounting for up to 25% of all cancer cases. Annually, an estimated 7-8000 new cases are diagnosed, and unfortunately, 80% of these are already at an inoperable stage at the time of detection, thereby ruling out surgery as an option, instead necessitating systemic oncological treatment (1). The average age of individuals at the onset typically ranges between 50 and 70 years (2).

Primary lung cancer prevention strategies emphasize the reduction or elimination of the risk factors. The most widely recognized predisposing factors include a family history of lung cancer; tobacco consumption in any form, including cigars, pipes, cigarettes, and secondhand smoke; exposure to other hazardous chemical substances, like radon, uranium, arsenic, asbestos, etc.; prior radiation therapy to the chest wall; outdoor air pollution, particularly from diesel exhaust; certain dietary factors, such as excessive beta-carotene, which paired with tobacco use can increase the risk of lung cancer (3).

The focus of secondary prevention of lung cancer is on detecting the disease in its early stages when treatment is more effective and slowing down the progression of the already existing disease. This approach is particularly important for screening individuals considered high-risk, such as long-term smokers or those with exposure to harmful substances. One of the key components of secondary prevention is the recognition of early symptoms, including persistent cough, especially with changes in its character, hemoptysis, chest pain, difficulty breathing, and general symptoms like unexplained weight loss, weakness, and fatigue. On the other hand, regular monitoring of risk factors is also equally important. An essential part of routine surveillance is the implementation of rapid diagnostic procedures when suspicious symptoms or screening results arise. Currently, the most effective screening method for the early detection of lung cancer is considered to be low-dose CT (LDCT), which can detect early-stage, asymptomatic lesions larger than 3 mm (4). It is recommended for individuals over the age of 50 who

have a smoking history: ≥15 cigarettes/ day for 25 years or ≥10 for over 30 years, former smokers who quit less than 10 years ago, or have other significant risk factors (5, 6).

For the success of secondary prevention, it is crucial to ensure proper public and patient education, where the at-risk populations are informed about the possibility of lung cancer screening and the importance of early detection (7, 8).

### 1.2. Lung adenocarcinoma (LADC)

Lung cancer can be classified into two main groups: non-small cell lung cancer (NSCLC), which accounts for nearly 85% of the cases, and small cell lung cancer (SCLC), which comprises the remaining 15%. LADC is the most prevalent type, as it constitutes nearly half of all NSCLC diagnoses. LADC can be further divided into invasive non-mucinous or invasive mucinous tumors. Based on the morphological phenotypes, we recognize five different subgroups, namely: solid, lepidic, acinar, papillary, and micropapillary (9). Notably, the solid and micropapillary subtypes are associated with a higher chance of developing early metastasis and are known to have poorer survival outcomes, whereas lepidic predominant adenocarcinomas generally correlate to a more favorable prognosis (10).

To develop a treatment plan for lung cancer, several factors must be considered, including the location of the primary tumor, its histological type, and the extent of the cancer (including the way of the tumor's spread), the presence of regional and distant metastases, and the overall health and surgical eligibility of the patient (11). The implemented therapy is determined by a multidisciplinary team, with consideration of the histological profile.

Before initiating treatment, molecular testing should be completed to identify alterations in the key biomarkers, for example, EGFR, ALK, KRAS, ROS1, BRAF, NTRK1/2/3, METex14 skipping, RET, and ERBB2 (HER2). The current clinical guidelines provide specific recommendations for appropriate treatment if a clinically actionable biomarker is identified (12).

A proper approach can be formulated based on the TNM classification (13). In cases deemed operable, surgical resection remains the primary method of treatment and typically involves an anatomical resection of the tumor and lymph node mapping

according to protocol (14, 15). The subsequent oncological treatment depends on both the genetic profile of the tumor and the disease stage. Platinum-based chemotherapy (CHT), possibly combined with targeted therapies and immunotherapy, offers promising results and improved clinical outcomes (16, 17).

Kirsten rat sarcoma viral oncogene (KRAS) is the most common oncogenic driver in NSCLC in the overall population. It most frequently occurs among the Caucasian and African-American patients, where it can be found in about one-third of cases (18). It is followed by Epidermal growth factor receptor (EGFR) mutations, which can be identified in about 10% to 20% of the patients (19). Notably, in Asian populations, EGFR mutations are more prevalent than KRAS mutations (20, 21).

KRAS belongs to the Ras GTPase family of proteins (22, 23), and similarly to the other Ras proteins, functions as a cellular switch. Upon activation by extracellular signals, such as growth factors, KRAS initiates the downstream signaling pathways. KRAS exhibits a slow nucleotide exchange rate and weak intrinsic GTPase activity. In approximately 75% of KRAS mutant LADCs, there is a heterozygous loss of the wild-type allele, leaving the mutant allele as the only functional form of KRAS, a state similar to homozygosity. Over 80% of KRAS mutations occur in exon 2 at codon 12, a region located near the nucleotide-binding pocket of the KRAS protein and adjacent to the effector protein switches. Among KRAS codon 12 mutations, the glycine-cysteine mutations (G12C) are the most common, accounting for approximately 40% of cases, followed by glycine-valine mutation (G12V) and glycine-aspartic acid mutation (G12D), both of which represent around 20%. G12C and G12V mutations are more prevalent in individuals with a history of smoking, while the transition mutation, which results in KRAS G12D, is more commonly found in never-smokers (24). Importantly, G12D and G12V mutations are associated with chromosomal instability and deficiencies in mismatch repair mechanisms. Regarding treatment response, platinum-based chemotherapies are generally effective for KRAS-mutant tumors, with the G12V variant showing an even higher sensitivity than the rest. Furthermore, the G12V and G12D mutations are associated with higher levels of immunotherapy biomarkers, indicating a better response to immune checkpoint inhibitors. However, G12C mutations correlate with positive programmed death-ligand 1 (PD-L1) expression, also suggesting a potential benefit from immunotherapy for this subgroup (25). Co-occurring driver mutations in LADC are relatively uncommon and

typically involve dual mutations of KRAS with EGFR, ALK, or BRAF. However, KRAS mutations most often appear as single driver mutations. Nevertheless, KRAS mutations frequently occur together with mutations in tumor suppressor genes such as TP53, STK11, and KEAP1/NFE2L2 (26), which may be associated with different tumor characteristics and biological behaviors (8).

The treatment of KRAS mutant LADC remains a challenge due to the considerable heterogeneity of KRAS mutations, including different genotypes and possible commutations, all of which influence the therapeutic response and clinical outcomes. Recent advances have led to the development of new therapeutic options that show promising results for the treatment of KRAS mutant lung cancer (27). Clinical trials have proved that covalent KRAS G12C inhibitors AMG510 (sotorasib) and MRTX849 (adagrasib) demonstrate a promising efficacy in KRAS G12C mutant LADC, both of these inhibitors target a specific mutation in KRAS, thus providing a new, more personalized treatment strategy (28).

Immunotherapy, particularly immune checkpoint inhibitors targeting the programmed cell death protein 1 (PD-1)/PD-L1 axis, has emerged as a promising approach for KRAS mutant NSCLC. The presence of an inflammatory tumor microenvironment (TIM) and the increased tumor immunogenicity associated with the KRAS mutation both contribute to the improved survival rates with the use of targeted therapies. Further ongoing studies are actively researching the combination of immunotherapy with other targeted treatment options, such as MEK and FGFR1 inhibitors, to overcome adaptive resistance and further enhance treatment efficacy (29). These developments underscore the need for more personalized approaches that also consider the biological diversity of the disease in the treatment of KRAS-mutant lung cancer. Current research efforts are focused on developing appropriate therapies by understanding the resistance mechanisms, optimizing combination therapies, and exploring the role of switch genes in modulating treatment response (30, 31).

Previously published studies have shown the variability of existing inflammation cells within the cancer and proved that tumor adaptation is strongly influenced by the development of the surrounding TIM. The understanding of TIM leads to identifying

biomarkers that are capable of distinguishing patient populations that could potentially benefit from current immune checkpoint blockade therapies (32).

Among the key components regulating tumor-associated inflammatory response are the inflammasomes, multiprotein complexes that regulate the immune system by activating inflammatory cytokines and thereby influence both tumor-promoting and tumor-suppressing pathways. They play an important role in tumor development and progression, and their study may contribute to the discovery of new therapeutic pathways to further improve treatment options.

The most extensively studied and characterized inflammasome is the NOD-like receptor family pyrin domain-containing 3 (NLRP3). NLRP3 functions as a cytosolic multiprotein complex that recognizes cytosolic cellular danger signals, promotes the maturation and secretion of proinflammatory cytokines, and has a role in mediating inflammatory responses. Upon activation, NLRP3 triggers the activation of the caspase-1 enzyme, which in turn releases proinflammatory cytokines, including IL-1β and IL-18, leading to pyroptotic cell death (33). NLRP3 activity can be modulated by the use of inhibitors, antagonists, and monoclonal antibodies. Thus, the targeted intervention in the NLRP3 inflammatory process could offer a new strategy in tumor treatment.

### 1.3. Lung neuroendocrine neoplasms (LNENs)

Neuroendocrine tumors constitute roughly 20 % of all lung cancers. LNENs originate from neuroendocrine cells in the lungs and are divided into four groups: typical carcinoid (TC), atypical carcinoid (AC), large cell neuroendocrine carcinoma (LCNEC), and SCLC (34). Each subtype has a distinct biological behavior, differing treatment options and prognosis.

LNENs can be further classified according to their level of differentiation and aggressiveness (35). TCs are well-differentiated and low-grade tumors, whereas ACs are considered to be intermediate-grade. LCNEC and SCLC are typically poorly differentiated, high-grade tumors (36). Based on new molecular knowledge of LNENs, the World Health Organization (WHO) revised the classification of LNENs in the 5th edition (9, 37, 38). The Ki-67 protein is a marker for cell division activity that indicates

the percentage of cells in the dividing phase in the examined tumor (39). The rate of cell division can be used to infer the level of tumor aggressiveness, which influences the applied treatment strategies, as tumors with a higher Ki-67 index tend to behave more aggressively and require a more intensive therapeutic regime (40, 41).

Treatment of LNENs might include surgery, chemotherapy, immunotherapy, and precision oncology treatments (42, 43). For localized low- or intermediate-grade LNEN, meaning TC or AC, the first choice is surgical treatment, which includes the anatomical resection with extended lymph node dissection (44). If the process is advanced, individually tailored, multidisciplinary treatment is recommended. Similar to treating SCLC, platinum-based chemotherapy is used for poorly differentiated neuroendocrine tumors in the form of cisplatin or carboplatin combined with etoposide or temozolomide (45). Additional effective therapeutic options for low-grade, metastatic LNENs include the administration of long-acting somatostatin analogues (SSAs), such as octreotide and lanreotide, or peptide receptor radionuclide therapy (PRRT) (46, 47). As an immunosuppressant, the mTOR inhibitor everolimus also improves outcomes in the treatment of advanced, progressive tumors (48-50). However, in high-grade poorly differentiated LNEN tumors, the use of immune checkpoint inhibitors as a monotherapy has shown limited success (51). Their combination with chemotherapy or targeted agents may overcome resistance and improve the efficacy of the therapy in selected patients. Preliminary evidence suggests that combination immunotherapy with nivolumab plus ipilimumab may enhance efficacy in treating LNENs (52).

## 2. Objectives

In the treatment of advanced LADC, alongside the traditionally used chemotherapy and radiotherapy, the role of immune checkpoint inhibitors (PD-1, PD-L1) has become increasingly significant (53). However, the therapeutic response to these agents varies considerably among individuals. Consequently, there is a clear need for predictive markers to determine the expected therapeutic response to specific treatments (54). In addition to immune checkpoint inhibitors, biomarkers such as tumor mutational burden and microsatellite instability have emerged as effective predictors of immunotherapeutic response (55). Comprehensive mapping of the tumor immunogenicity and immunophenotype is thus essential in determining the appropriate therapeutic strategies (56, 57).

Genetic mutations, such as KRAS alterations in LADC, are known to affect several signaling pathways and enhance cell proliferation (58). These signaling pathways also impact the TIM by triggering inflammatory processes (59). Different KRAS mutant tumors have a distinct response to immunotherapy (60, 61). A detailed analysis of the cytokines, regulatory proteins, and effector immune cells involved in tumor-associated inflammation may provide valuable insight into the relationship between inflammation and immunomodulation induced by KRAS mutations (62). One of the key regulators of the hereditary inflammatory response is the NLRP3 inflammasome, which can be activated through specific signaling pathways (63, 64). Overactivation of NLRP3 leads to increased production of pro-inflammatory cytokines. Thus, NLRP3 inhibitor therapy may offer a novel approach to managing KRAS mutant adenocarcinomas (33, 65).

Typical and atypical carcinoids, although relatively rare subtypes of neuroendocrine lung tumors, are of growing importance (66). TC accounts for 2% of lung tumors, while AC accounts for about 0.2%, but their incidence is increasing worldwide (67). TC tumors are generally associated with better prognosis and longer overall survival (OS) compared to AC tumors, which demonstrate more aggressive clinical behavior (68). In cases of metastatic carcinoid tumors, occurring in 10-20% of TCs and 40-50% of ACs, therapeutic options remain limited, mainly due to the high resistance of these tumors to radiotherapy and chemotherapy (69, 70). The identification of new therapeutic targets for treating malignancies is therefore essential. While several clinical trials are currently

exploring immunotherapeutic approaches for LNEN, another cancer classified as a neuroendocrine tumor, these trials have thus far generated many unanswered questions (71-74). As a result, progress remains slower compared to the advancements seen in NSCLC (75, 76).

Our study aims to explore the immunological profile and immunophenotype in relation to molecular characteristics and clinicopathological variables in malignant KRAS-mutant LADC and LNEN.

# 3. Methods

## 3.1. KRAS -study

### 3.1.1. Population and treatment

This retrospective study included 87 patients diagnosed with early-stage LADC, classified as stage I. A to II.B, all of whom had their diagnosis histologically confirmed. These patients underwent surgical resection at the National Koranyi Institute of Pulmonology in Budapest, Hungary, between 2012 and 2017. The selection criteria required the presence of a presumed KRAS mutation identified through previous direct sequencing.

During clinical data collection, we retrospectively processed the data obtained from the hospital's medical records on the patient's age, gender, comorbidities, and smoking habits at the time of diagnosis. Patient identifiers were recorded to ensure that the individuals included remained pseudonymous. Due to the retrospective nature of the study, informed consent was not required. Therefore, no signed forms were obtained from the participants. The study adhered to the guidelines of the Helsinki Declaration by the World Medical Association and received approval from the National Ethics Committee of Hungary (Hungarian Scientific and Research Ethics Committee of the Medical Research Council, ETT-TUKEB 23636-2/2018, 23636/10/2018/EÜIG).

### 3.1.2 Immunohistochemistry (IHC)

To analyze morphological heterogeneity, tumor tissue samples were obtained through surgical resection, and three tissue microarray (TMA) punctures were extracted from each formalin-fixed, paraffin-embedded (FFPE) block. TMA tissue cores were retrieved from distinct representative regions within viable tumor areas. The TMA blocks were cut into four-micron-thick sections and stained with hematoxylin and eosin (H&E) and alcian blue to highlight histological patterns.

The extent of tumor-associated lymphocyte (TIL) and macrophage infiltration in the peritumoral region was assessed through immunostaining for CD3 and CD163, according to the scoring system established by the International TILs Working Group in 2014 (77). Additionally, to obtain a comprehensive overview of inflammasome expression in the

specimens, each slide was stained for NLRP3. The following antibodies were used for IHC staining: CD3 (Leica, rabbit monoclonal antibody, clone number LN10, 1:200 dilution), CD163 (Invitrogen, rabbit monoclonal antibody, clone number MRQ-26, 1:200 dilution), and NLRP3 (Invitrogen, rabbit monoclonal antibody, clone number SC06-23, 1:200 dilution) (Table 1).

**Table 1. Antibodies used for immunohistochemistry (IHC).**

| Antibody | Company | Catalog nr. | Host | Dilution | Antigen retrieval |
|---|---|---|---|---|---|
| **TIM3** | Abcam, Boston, USA | ab185703 | Rabbit | 1:100 | Citrate (pH=6.0) |
| **VISTA** | Sino Biological, Beijing, China | 13482-T24 | Rabbit | 1:500 | Citrate (pH=6.0) |
| **GITR** | Thermo Fisher Scientific, Waltham, MA, USA | PA5-46810 | Rabbit | 1:100 | Citrate (pH=6.0) |
| **OX40L** | Thermo Fisher Scientific, Waltham, MA, USA | 11-1347-42/ACT35 | Rabbit | 1:100 | Citrate (pH=6.0) |
| **CD3** | Leica, Wetzlar, Germany | PA0553/LN10 | Mouse | Ready-to-use | Tris-EDTA (pH=9.0) |

All staining procedures were conducted following the manufacturer's protocols using the fully automated BenchMark ULTRA IHC/ISH system (Roche Diagnostics, Rotkreuz, Switzerland). Antibody binding was detected using the ImmPACT DAB Substrate Kit from Vector Laboratories, and the nuclei were counterstained with hematoxylin. All antibodies were validated by utilizing appropriate tissue controls. The expression levels of the specific markers were assessed on a categorical, semi-quantitative scale by two experienced pulmonary pathologists who were blinded to the clinical data. In addition to the analysis of tumorous lesions, one pathologically verified non-tumorous lung tissue core was retrieved from each patient for control purposes. We performed IHC and molecular analyses on both tumor and non-tumor samples.

### 3.1.3. Molecular analyses

We analyzed the KRAS mutation status of both tumor and associated non-tumorous controls using Competitive Allele-Specific (CASP) TaqMan Mutation Detection Assays

using real-time PCR (RT-PCR). A tumor cell-rich 2 mm cylinder, marked by the pathologist, was extracted from the FFPE tissue specimen using a TMA Master (3DHistec) tissue microblock maker. The samples were deparaffinated using Deparaffinization Solution from Zymo Research. DNA isolation was performed using a King Fisher Duo Prime (Thermo Scientific) automated nucleic acid purifier and the MagMAX FFPE DNA/RNA Ultra Kit (Applied Biosystems). The following seven KRAS mutation subtypes were consecutively investigated using the CASP TaqMan Mutant Allele Assay: G12D (Hs00000121_mu), G13D (Hs00000131_mu), G12V (Hs00000119_mu), G12R (Hs00000117_mu), G12A (Hs00000123_mu), G12S (Hs00000115_mu), and G12C (Hs00000113_mu). Additionally, a KRAS gene reference assay (Hs00000174_rf) designed for the mutation-free region of the KRAS gene was included for each sample. In our study, RT-PCR reactions were performed in 96-well plates according to the manufacturer's protocol, which was used for the quantification of the mutant allele frequency in the samples.

### 3.1.4. Statistical analysis

We used a pairwise chi-square test to examine the relationships between categorical variables. Since none of the results were statistically significant even before adjusting for multiple testing, no corrections were applied in this analysis. While expression levels were originally defined on a categorical scale (none/ slight, diffuse plasma staining/ medium plasma staining with dominant spots/ strong diffuse plasma staining), they could be converted into semi-quantitative numerical values (e.g., 0/1/2/3) based on their ordinal nature. This conversion allowed for a reassessment of relationships between different expression levels using Pearson correlation and between expression levels and categorical variables using pairwise t-tests adjusted for multiple testing with the Bonferroni correction. Additionally, when comparing different expression levels, a simpler categorization of "none" versus "any" was applied, and a Fisher's exact test was used to determine whether the different types of expressions were independent. For specific analyses, tumor tissue cores were treated independently, even if obtained from the same patient. For each patient, we examined pairs of samples from the tumor and the adjacent nontumor environment to map their mutational status and expression levels. The samples from the three tumor sites were compared to the same normal sample. Survival curves for

different patient groups were estimated using Kaplan-Meier plots. The differences between the groups were analyzed using the log-rank test. OS was defined as the time in months from the surgical intervention to the last available follow-up or the date of death from any cause. R version 4.4.1 was used for all statistical analyses, and a p-value of less than 0.05 was considered statistically significant.

### 3.2. LNEN- study

#### 3.2.1. Population and treatment

Four Central European centers participated in the second LNEN study: the National Koranyi Institute of Pulmonology, Budapest, Hungary, the National Institute of Oncology, Budapest, Hungary; the Medical University of Graz, Austria; and Palacky University, Olomouc, Czech Republic. A total of 141 patients who underwent surgical resection for LNEN between 1997 and 2021 were examined. The distribution of patients based on LNEN subtypes was as follows: 66 patients had SCLC, 49 LCNEC, and 26 AC. Only whole tissue samples were processed to avoid bias due to intratumoral heterogeneity. Before inclusion in the study, all sections associated with an LNEN diagnosis had to be re-examined to confirm the LNEN diagnosis. A board-certified pathologist at each center had reviewed FFP blocks to determine whether the sample contained adequate tumor content, defined as more than 20% of all cells being tumorous, and thus met the inclusion criteria.

All studies were conducted according to the guidelines of the World Medical Association Declaration of Helsinki and were approved by the respective ethics committees of each participating country. The national-level ethics committee of Hungary was Hungarian Scientific and Research Ethics Committee of the Medical Research Council, ETT TUKEB 39249–2/2019/EKU and 52614–4/ 2013/EKU. Patient identifiers were removed after collecting the clinical data to ensure the pseudonymity of the patients.

#### 3.2.2. Immunohistochemistry

The tissue samples were analyzed for the expression of four TIM markers, namely T cell immunoglobulin and mucin domain 3 (TIM3), V-domain immunoglobulin suppressor of

T cell activation (VISTA), Glucocorticoid induced tumor necrosis factor receptor (GITR), and Tumor necrosis factor receptor superfamily, member 4 (OX40L). Due to the limited quantity of samples, only VISTA expression was measured in 21 cases of SCLC. To evaluate the quality and reliability of older FFPE blocks (over 15 years) from SCLC patients, these samples were stained with commonly used diagnostic antibodies against CD56 (78) and Ki-67 (79). The level of immune infiltration was assessed by examining CD3 expression. Immunohistochemistry staining was performed following the recommended protocols using the Ventana BenchMark Ultra IHC/ISH System (Roche Diagnostics, Basel, Switzerland). Following deparaffinization and incubation with the primary antibody, a secondary antibody was applied for one hour at room temperature. The expression levels were visualized using the Liquid DAB and Substrate Chromogen System, and the sections were subsequently counterstained with hematoxylin. To ensure the reliability of the staining protocol, human tonsils were utilized as positive tissue controls. All slides were digitally scanned using the PANNORAMIC 250 Flash III (3DHISTECH Ltd., Budapest, Hungary), and the sections were thoroughly examined and evaluated with CaseViewer 2.4 (3DHISTECH Ltd., Budapest, Hungary).

It is important to provide an overview of generalized marker expression throughout the tumor area for effective biomarker discovery (80). Therefore, during the pathological evaluation, the percentage of positive tumor cells was determined in at least 20 randomly selected areas at 20× and 40× magnifications. Two experienced pulmonary pathologists undertook the evaluation process. A third pulmonary pathologist was consulted to provide additional insight when encountering a discrepancy greater than 20% between their findings. Tumor cells were evaluated separately from immune cells.

For tumor cells, we quantified the ratio of positive cells to the total number of tumor cells. Similarly, we assessed the ratio of immune cells with positive staining to the total immune infiltrates in each sample.

In our study, the analysis of individual markers has been carried out manually, given that doubts arise regarding software-based assessments even in the case of antibodies used in routine diagnostics.

### 3.2.3. Statistical analysis

All statistical analyses were performed with R version 4.2.1 (R Foundation for Statistical Computing, Vienna, Austria). Fisher's exact tests and Kruskal-Wallis rank sum tests were used to assess associations between histological subtypes and clinicopathological characteristics, specifically for categorical and continuous variables. The Bonferroni method was applied to adjust for multiple comparisons. Marker expression levels and clinicopathological parameters were compared using Wilcoxon signed-rank tests, with Bonferroni correction applied. Hierarchical clustering of samples based on expression levels was performed using the Complex Heatmap R package (version 2.10.0). The distance matrix was calculated using the Manhattan distance measure, and dendrograms were created using the ward.D clustering method. During the statistical analysis of the obtained results, Pearson correlation coefficients (R) were calculated between expression levels, and the Bonferroni method was used to correct p-values for multiple comparisons.

Furthermore, we wanted to know which expression levels are the strongest indicators of the LNEN subtype, for which we performed principal component (PC) analysis using the factoextra R package (version 1.0.7). This analysis aimed to find linear combinations, referred to as PCs of the measured variables (expression levels), that most effectively explain the variance in the data. Univariate Kaplan-Meier analyses were used to determine the clinical factors with prognostic relevance for OS, and survival curves of different patient subgroups were compared using log-rank tests without adjusting p-values for multiple testing. Patients were categorized into "low" (i.e., median or below-median) and "high" (i.e., above-median) expressing groups based on the median expression value of each marker. A multivariate Cox regression model was constructed based on the results of the univariate analyses to evaluate the prognostic significance of various marker expressions.

# 4. Results

## 4.1. KRAS- study

### 4.1.1. Association of tumor morphology and KRAS mutational status

Pathological evaluation of tumor cores revealed a single dominant morphological component in 71% of cases. As shown in Figure 1A, 47% of samples showed an acinar pattern, followed by solid (26%), lepidic (22%), and papillary architectures (5%). Notably, 23% of all tumors displayed two dominant morphological components, while tumors with three components occurred in 6% of cases. Next, we assessed the KRAS mutational status for all TMA specimens (Figure 1B). Regarding mutation subtypes, KRAS G12C, KRAS G12D, and KRAS G12V were the most frequently detected genetic alterations in our cohort, found in 33%, 25%, and 24% of all patients, and in 38%, 23%, and 24% of the samples, respectively. It is important to note that while the mutational landscape concerning KRAS mutations was mostly homogeneous across different TMA cores from the same tumor, there were 19 cases where the dominant mutational subtype varied between tumor punctures. Moreover, 14 LADC samples completely lacked any KRAS mutations despite the initial diagnosis of KRAS mutant LADC established by direct sequencing. This finding further underscores that KRAS mutant lesions might be highly heterogeneous, even within a single tumor.

We also studied the distribution of KRAS mutation subtypes between the different morphological patterns (Figure 1C). Notably, the KRAS G12A mutation was absent in lepidic LADCs. In contrast, the micropapillary LADC samples did not contain wild-type KRAS genes or multi-hit (i.e., simultaneous multiple types of) KRAS alterations. Despite these obvious differences, the KRAS mutational status did not show a statistically significant association with the morphological growth patterns. Furthermore, the evaluation of mucin secretion revealed that 33.3% of tumors do not express mucin at all. Intracellular mucin secretion was found in 11.5% of cases, while extracellular and mixed mucin secretion was observed in 43.7% and 11.5% of the examined LADCs, respectively. Additionally, mucin secretion was not influenced by KRAS mutational status.

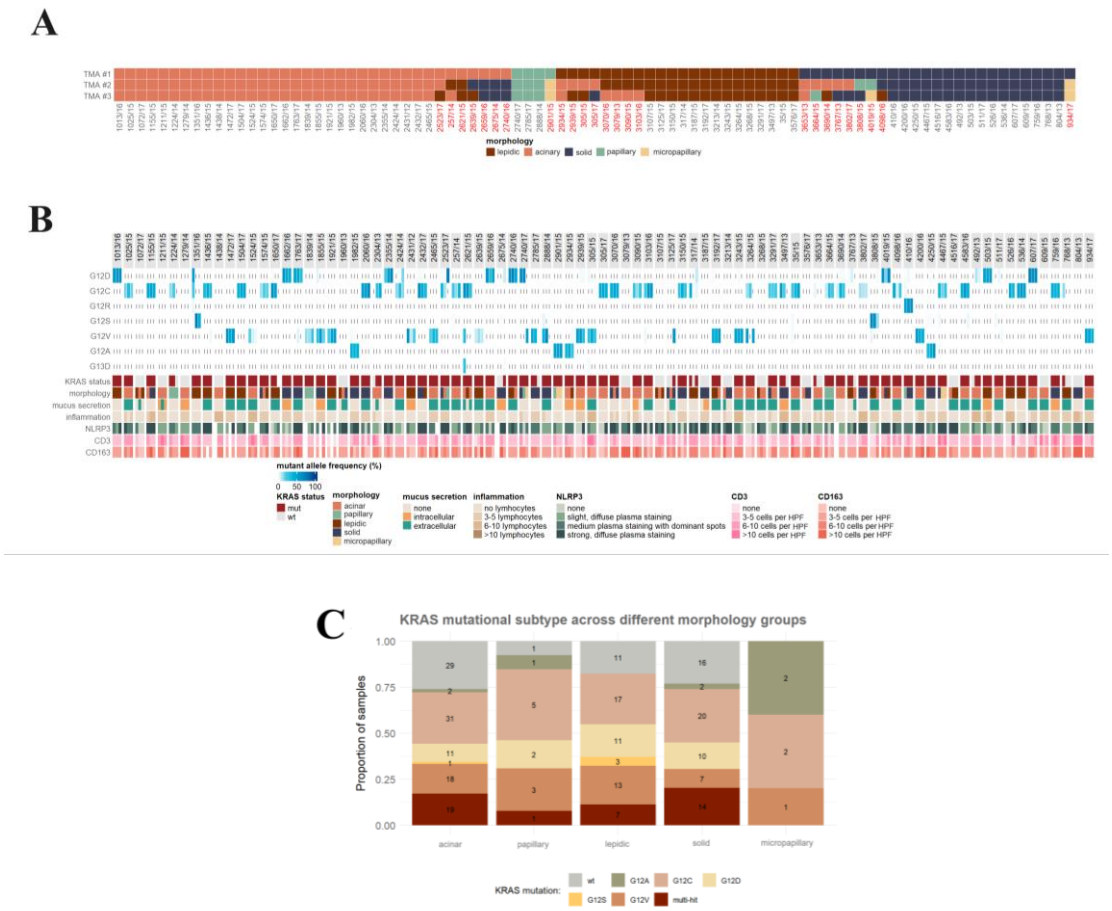


**Figure 1. Morphological and mutational features of surgically resected LADC samples.** (***A***) Histological growth pattern of included samples. Patients are represented as columns, while their three separate tumor tissue cores (i.e., TMAs #1, #2, and #3) are arranged as rows. Three distinct tumorous TMA cores were analyzed from each surgically resected LADC. (***B***) Heatmap of KRAS mutation status of different samples with regards to morphological features such as tumor content, growth pattern, mucus secretion, inflammation, and NLRP3, CD3, CD163, and PD-L1 expression. Patients are represented as columns, with the tumor samples belonging to each patient indicated as thin stripes within these. The color bar scale indicates the expression levels of the selected markers. 14 out of 87 patients had KRAS wild-type tumors across all three tumor tissue cores. Additionally, 12 more patients had at least one tumor tissue core identified as KRAS wild-type. This adds up to a total of 57 KRAS wild-type tumor tissue cores out of the total of 261 examined tumor tissue cores (three per patient). These KRAS wild-type samples are marked in grey in the "KRAS status row" of the corresponding heatmap (***C***) Association of dominant growth pattern and KRAS mutational status of surgically resected LADC

tumor tissue cores. Tumor tissue cores were treated independently, even if they were obtained from the same patient. LADC, lung adenocarcinoma; HPF, high power fields; Mut, mutated; wt, wild-type; TMA, tissue microarray.

As shown in Figure 2, patients with KRAS wild-type tumors tended to have better survival outcomes than those with specific KRAS mutations, yet differences in median OS did not reach statistical significance (p=0.138). Tumors that exhibited heterogeneous KRAS mutational status across different tissue cores were linked to improved survival (vs. homogenous tumors; median OSs were 52 months vs. 39 months, respectively; p=0.081), with borderline significant statistical outcomes. Given the relatively small cohort size, results related to OS should be interpreted with caution.

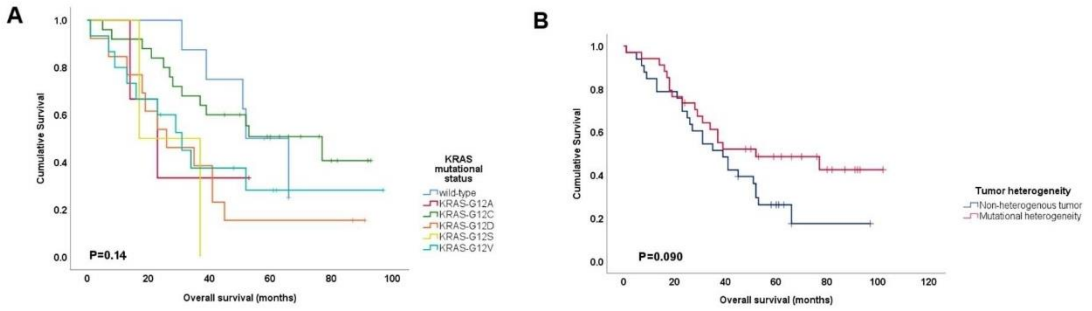


**Figure 2. Kaplan-Meier estimates for OS according to (*A*) KRAS mutational status and (*B*) KRAS mutational status-related tumor heterogeneity.** Heterogeneous tumors were defined as LADC samples with a heterogeneous KRAS mutational landscape across different tumor tissue cores. Survival data was not available in 18 cases. OS, overall survival.

### 4.1.2. NLRP3 expression is associated with increased immune infiltration

The morphological growth patterns of the examined LADCs did not appear to influence the distribution of NLRP3 when the surgically resected specimens were categorized according to the predefined NLRP3 expression subgroups (Figure 3A). However, when NLRP3 expression was evaluated on a semi-quantitative scale, solid LADCs demonstrated significantly higher levels of NLRP3 compared to acinar samples (p=0.001) (Figure 3B). Although the PD-L1 group showed no correlation with tumor morphology

(Figure 3C), when evaluating PD-L1 expression on a continuous scale, we observed that PD-L1 expression was significantly higher in tumor tissue cores with solid morphology than in those with acinar (means: 14.6% vs. 4.4%, t-test adjusted p-value: 0.007) or lepidic growth patterns (means: 14.6% vs. 1.6%, t-test adjusted p-value: 0.002) (Figure 3D). Representative IHC images of NLRP3, CD3, CD163, and PD-L1 expression are shown in Figure 4.

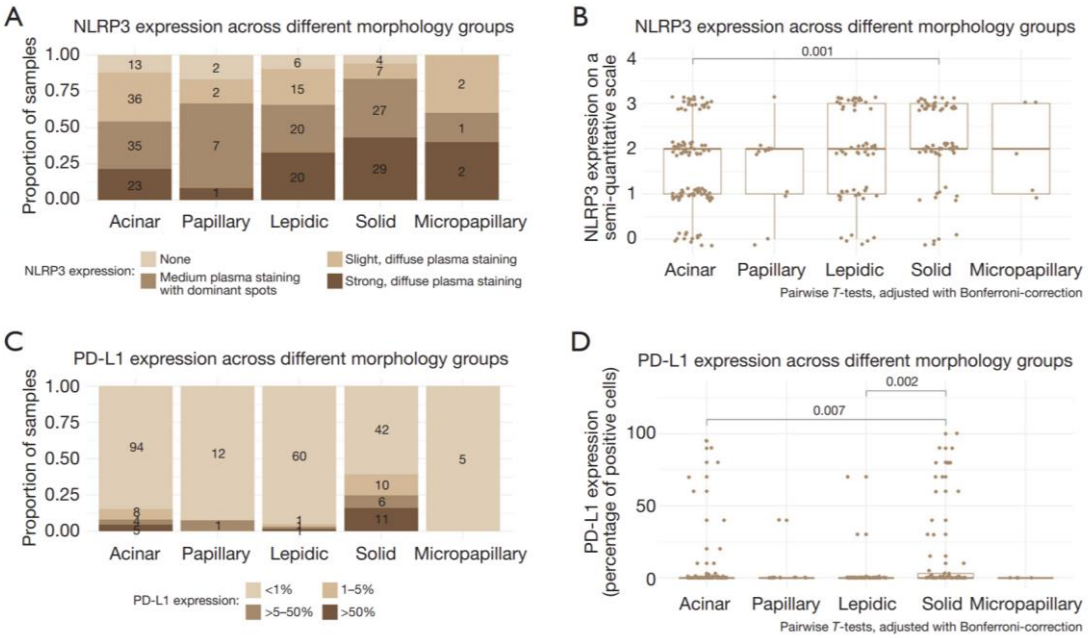


**Figure 3. Association of NLRP3 and PD-L1 expression with tumor morphology in LADC tumor tissue cores.** Distribution of growth patterns according to (***A***) NLRP3 and (***C***) PD-L1 subgroups. (***B***) NLRP3 and (***D***) PD-L1 expression on a semi-quantitative scale across different morphology groups.

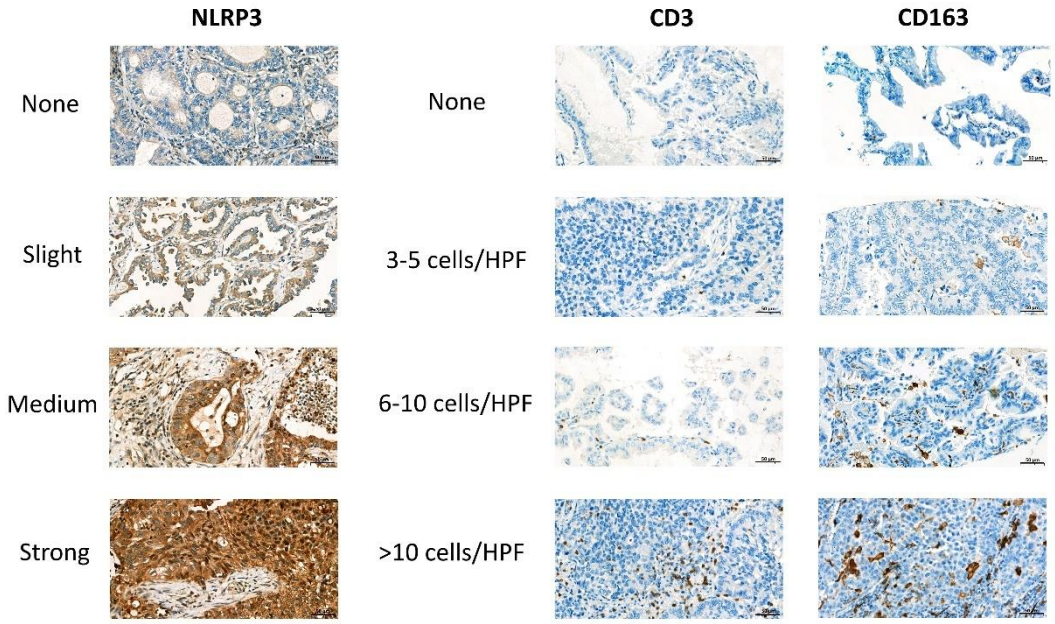


**Figure 4. IHC staining of formalin-fixed, paraffin-embedded LADC samples with markers of the immune microenvironment (NLRP3, CD3, CD163, and PD-L1)**. The representative images were captured with a 40x objective lens. The positive cells were visualized with 3-3′-diaminobenzidine (DAB), and the nuclei were labelled with hematoxylin. IHC, immunohistochemistry; LADC, lung adenocarcinoma; HPF, high power field.

The abundance of CD3 T lymphocytes, defined as the number of CD3 cells per high power field (HPF) according to NLRP3 expression, is illustrated in Figure 5A. Notably, a statistically significant weak positive linear correlation between CD3 and NLRP3 expression on a semi-quantitative scale ($R = 0.31$, $p < 0.0001$) was found, as shown in Figure 4B. We categorized the samples into groups based on the expression (or lack of it) of CD3 and NLRP3 and discovered that NLRP3-expressing LADC samples most frequently exhibited detectable CD3 expression ($p < 0.0001$, Figure 5B). In contrast, tumors without NLRP3 expression displayed a CD3-depleted phenotype. Regarding macrophage abundance, the number of CD163 cells per HPF was considerably higher in samples with NLRP3 plasma staining than those lacking NLRP3 expression, as indicated in Figure 5C. We also observed a statistically significant positive linear correlation

between CD163 and NLRP3 expression (Figure 5D). In addition, LADC samples expressing NLRP3 generally tended to also express CD163.

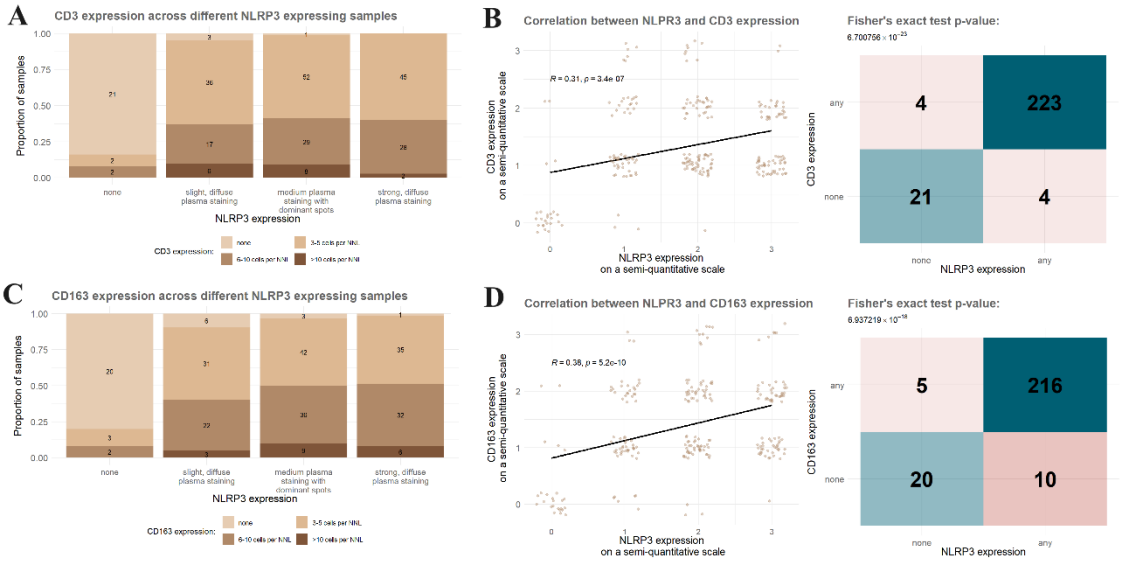


**Figure 5. NLRP3 is associated with immune infiltration as defined by CD3 and CD163 expressions.** (***A***) CD3 expression across different NLRP3-expressing groups in tumor tissue cores. (***B***) Correlation between NLRP3 and CD3 expression on semi-quantitative scales combined with a Fisher's exact test. (***C***) CD163 expression across different NLRP3-expressing groups in tumor tissue cores. (***D***) Correlation between NLRP3 and CD163 expression on semi-quantitative scales combined with a Fisher's exact test. HPF, high power field.

Moreover, we also found that LADC samples showing NLRP3 expression tended to have CD163 expression as well. PD-L1 expression was generally higher in tumor tissue cores with medium or strong NLRP3 staining; however, no statistically significant associations were found between PD-L1 expression and NLRP3, CD3, and CD163 levels (Figure 6).

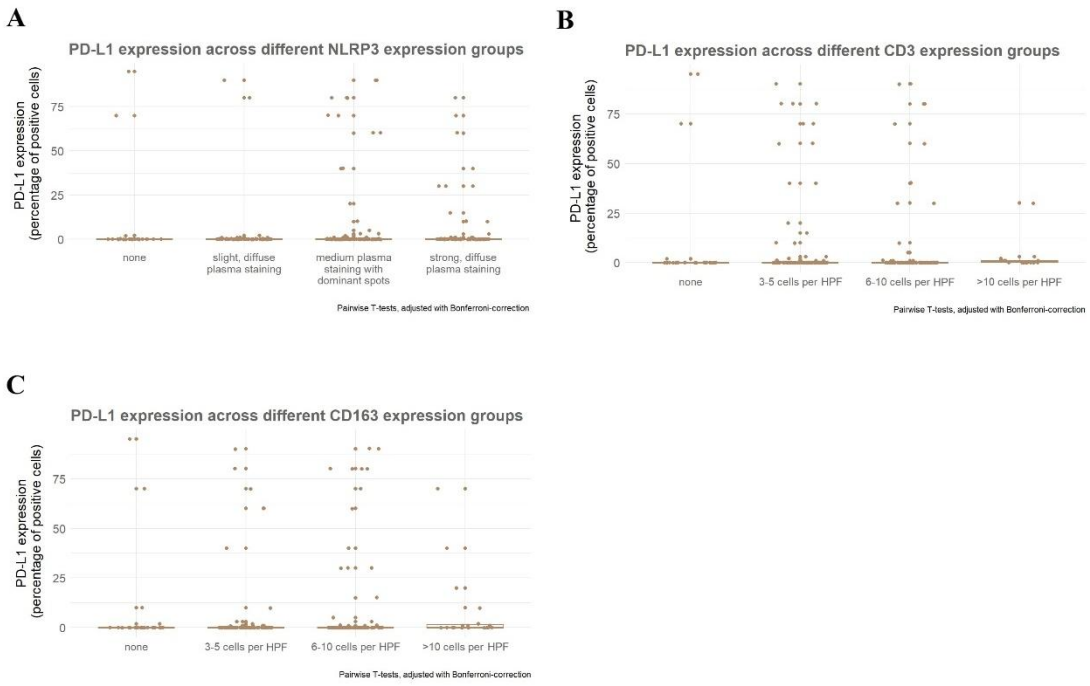


**Figure 6. PD-L1 expression across different (*A*) NLRP3, (*B*) CD3, and (*C*) CD163 expression subgroups.** HPF, high power field.

### 4.1.3. Relation between KRAS mutational subtype and NLRP3 expression

As shown in Figure 7A, the most frequently detected KRAS mutation subtype in both NLRP3 expressing and NLRP3 non-expressing tissue cores was KRAS G12C. However, the second most common KRAS mutation subtype differed between the two groups, with KRAS G12V found in NLRP3 expressing tumors and KRAS G12D in NLRP3 non-expressing tumors. However, Figure 7B also shows that we did not find a statistically significant correlation between KRAS mutation subtypes and NLRP3 expression when examining expression levels on a semiquantitative scale. PD-L1 expression was not associated with KRAS mutation status (Figure 7C and 7D).

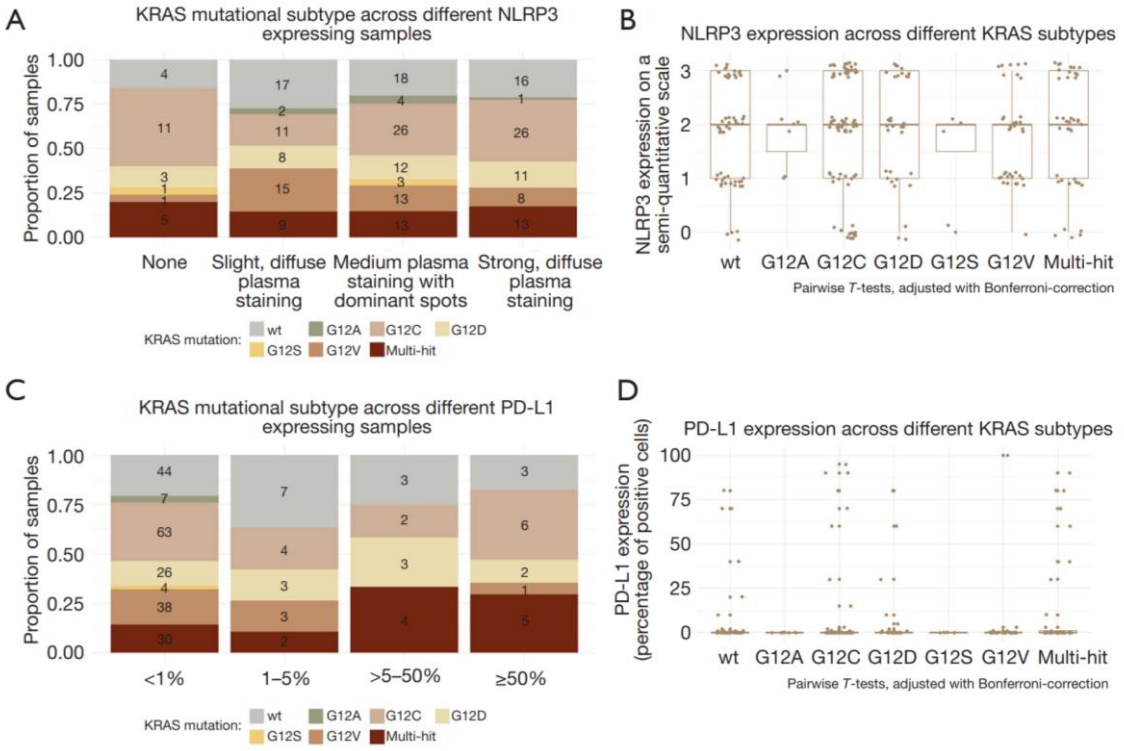


**Figure 7. Comparative analysis of KRAS mutational landscape and NLRP3 and PD-L1 expression in surgically resected LADC tumor tissue cores.** KRAS mutational subtypes with regards to (***A***) different NLRP3 subgroups and (***B***) NLRP3 expression on a semi-quantitative scale. NLRP3 expression data were not available in 2 KRAS wild-type tissue cores. Distribution of KRAS mutation subtypes according to (***C***) PD-L1 subgroups and (***D***) PD-L1 expression on a semi-quantitative scale. Colors indicate different mutational statuses. LADC, lung adenocarcinoma.

### 4.1.4. KRAS mutation- and NLRP3 expression-based comparison of tumorous and adjacent non-tumorous tissue cores

We conducted a comparison between tumor tissue samples and non-tumorous control tissue sourced from the same patient. Each patient had a single non-tumorous tissue core and three paired sections of tumorous tissue analyzed. Although non-tumorous tissue specimens generally lack oncogenic driver mutations, we found that 20 out of 83 non-tumorous lung tissues (24.1%) exhibited KRAS mutations. Importantly, nearly half of the tumor tissue samples with the KRAS G12D mutation displayed a corresponding KRAS G12D mutation in their paired non-tumorous tissue samples (Figure 8A). Notably, nine tumor samples (3.61%) presented with wild-type KRAS status, while their corresponding

non-tumorous counterparts' cores revealed mutations in the KRAS gene. NLRP3 staining was observed in only a few cases of non-tumorous samples, and even in the cases where NLRP3 expression was noticeably higher, it did not alter the level measured in tumor tissue. This may indicate no close relationship between NLRP3 expression levels in tumor and non-tumorous tissues (Figure 8B). Most control samples displayed medium-level CD3 expression, while those with low CD3 expression tended to have a corresponding low-to-medium abundance of CD3 T cells in the associated tumors (Figure 8C). In tumor samples characterized by high CD3 expression, the corresponding non-tumorous specimens consistently demonstrated at least a medium level of CD3 expression, with a count of 3 to 10 cells per HPF. When examining CD163 macrophages, a statistically significant but weak positive linear correlation emerged between the tumor and non-tumorous lesions' expression levels ($R = 0.16$, $p = 0.01$; Figure 8D).

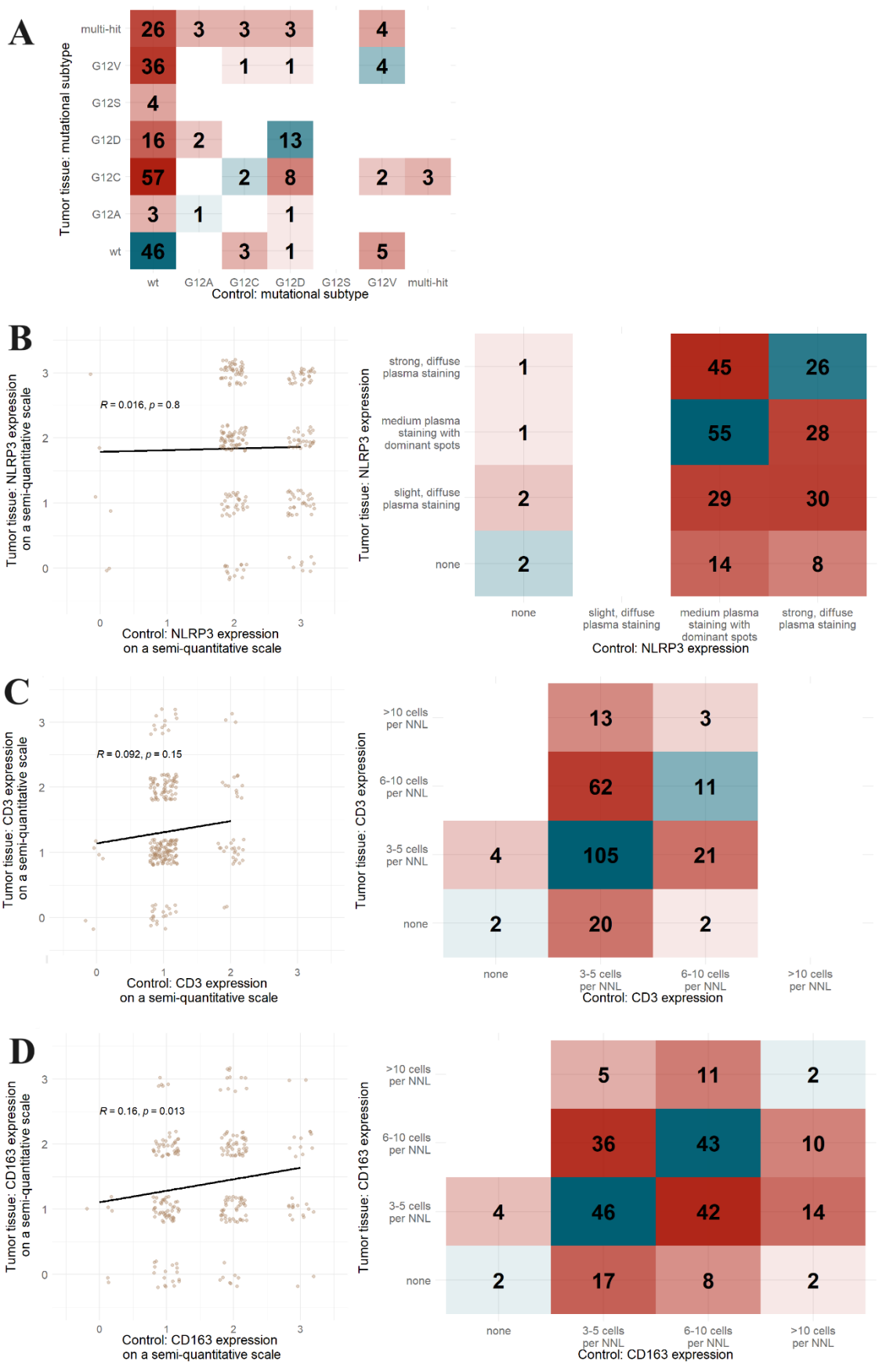
A
Tumor tissue: mutational subtype
multi-hit
G12V
G12S
G12D
G12C
G12A
wt
Control: mutational subtype
wt
G12A
G12C
G12D
G12S
G12V
multi-hit
B
Tumor tissue: NLRP3 expression on a semi-quantitative scale
R = 0.016, p = 0.8
Control: NLRP3 expression on a semi-quantitative scale
strong, diffuse plasma staining
medium plasma staining with dominant spots
slight, diffuse plasma staining
none
Control: NLRP3 expression
C
Tumor tissue: CD3 expression on a semi-quantitative scale
R = 0.092, p = 0.15
Control: CD3 expression on a semi-quantitative scale
>10 cells per NNL
6-10 cells per NNL
3-5 cells per NNL
none
Control: CD3 expression
D
Tumor tissue: CD163 expression on a semi-quantitative scale
R = 0.16, p = 0.013
Control: CD163 expression on a semi-quantitative scale
>10 cells per NNL
6-10 cells per NNL
3-5 cells per NNL
none
Control: CD163 expression

**Figure 8. KRAS mutation status and inflammatory characteristics of tumorous and adjacent non-tumorous tissue cores.** (***A***) Association between the tumorous and non-tumorous tissue cores' KRAS mutational status. Interestingly, nine tumor samples (bottom row) had wild-type KRAS status, while their paired non-tumorous tissue core displayed KRAS mutation. (***B, C, D***) Comparative analysis of tumorous and non-tumor tissue cores with regards to (***B***) NLRP3, (***C***) CD3, and (***D***) CD163 expression. All panels treat "sample pairs" as units, whether represented as points on scatter plots or numbers in cells. A "sample pair" is defined as the normal sample and the tumor sample from a specific patient. Most patients had one normal sample ($Nor_1$) and three tumor tissue cores ($Tu_1$, $Tu_2$, $Tu_3$), resulting in three "sample pairs" (i.e., $Nor_1$-$Tu_1$, $Nor_1$-$Tu_2$, $Nor_1$-$Tu_3$). Therefore, the numbers on the heatmaps do not sum up to either the total number of patients or the total number of individual tissue cores. HPF, high power field.

## 4.2. LNEN- study

### 4.2.1. Patient and sample characteristics

Patients in our LNEN study were analyzed according to clinicopathological characteristics, which are presented in Table 2, organized by subgroups. The majority of SCLC and LCNEC patients were active smokers, while the majority of patients diagnosed with AC were never smokers ($p<0.001$). According to the location of the tumor nest in the lung, we observed that SCLC tumors were more often located in the central part, while LCNEC tumors tend to be located peripherally ($p < 0.001$). We used univariate models to examine different clinicopathological factors to better understand the survival outcomes.

Factors such as diabetes, histological subtype, vascular involvement, and T and N stages were proven to be significant for survival outcomes. The conducted quality check on older FFPE samples (over 15 years old) found strong positivity with CD56 and moderate positivity with Ki-67. However, the expression patterns of TIM3, VISTA, GITR, and OX40L did not differ statistically between the older samples (>15 years) and the newer ones (≤15 years).

**Table 2. Clinicopathological characteristics of the study population.** Statistically significant p values are marked with bold.

| | | Total | AC | LCNEC | SCLC | p-value |
|---|---|---|---|---|---|---|
| **Total number of patients** | | 141 | 26 | 49 | 66 | |
| **Gender** | N/A | 9 | 0 | 6 | 3 | 0.771[1] |
| | Male | 66 (50%) | 11 (43.2%) | 23 (53.3%) | 32 (50.8%) | |
| | Female | 66 (50%) | 15 (57.7%) | 20 (46.5%) | 31 (49.2%) | |
| **Age** | N/A | 10 | 0 | 7 | 3 | 0.333[2] |
| | Median (Range) | 65 (33-79) | 62.5 (33-79) | 64.5 (41-78) | 65 (44-78) | |
| **Smoking status** | N/A | 36 | 2 | 13 | 21 | **<0.001[1]** |
| | Never | 21 (20.0%) | 14 (58.3%) | 3 (8.3%) | 4 (8.9%) | |
| | Ex | 44 (41.9%) | 6 (25.0%) | 15 (41.7%) | 23 (51.1%) | |
| | Current | 40 (38.1%) | 4 (16.7%) | 18 (50.0%) | 18 (40.0%) | |
| **COPD** | N/A | 13 | 0 | 8 | 5 | **0.049[1]** |
| | No | 77 (60.2%) | 22 (84.6%) | 21 (51.2%) | 34 (55.7%) | |
| | Yes | 51 (39.8%) | 4 (15.4%) | 20 (48.8%) | 27 (44.3%) | |
| **Hypertension** | N/A | 12 | 0 | 7 | 5 | 0.495[1] |
| | No | 55 (42.6%) | 10 (38.5%) | 15 (35.7%) | 30 (49.2%) | |
| | Yes | 74 (57.4%) | 16 (61.5%) | 27 (64.3%) | 31 (50.8%) | |
| **Diabetes** | N/A | 12 | 0 | 7 | 5 | 0.495[1] |
| | No | 104 (80.6%) | 22 (84.6%) | 36 (85.7%) | 46 (75.4%) | |
| | Yes | 25 (19.4%) | 4 (15.4%) | 6 (14.3%) | 15 (24.6%) | |
| **Tumor localization (central/peripheral)** | N/A | 22 | 0 | 10 | 12 | **<0.001[1]** |
| | Central | 51 (42.9%) | 13 (50.0%) | 5 (12.8%) | 33 (61.1%) | |
| | Peripheral | 68 (57.1%) | 13 (50.0%) | 34 (87.2%) | 21 (38.9%) | |
| **Tumor localization (upper/lower lobe)** | N/A | 41 | 0 | 7 | 34 | 0.333[1] |
| | Upper lobe | 72 (72.0%) | 15 (57.7%) | 32 (76.2%) | 25 (78.1%) | |
| | Lower lobe | 28 (28.0%) | 11 (42.3%) | 10 (23.8%) | 7 (21.9%) | |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Necrosis** | N/A | 22 | 10 | 7 | 5 | 0.333[1] |
| | No | 43 (36.1%) | 6 (37.5%) | 11 (26.2%) | 26 (42.6%) | |
| | Yes | 76 (63.9%) | 10 (62.5%) | 31 (73.8%) | 35 (57.4%) | |
| **Vascular involvement** | N/A | 16 | 1 | 7 | 8 | 0.771[1] |
| | No | 77 (61.6%) | 15 (60.0%) | 28 (66.7%) | 34 (58.6%) | |
| | Yes | 48 (38.4%) | 10 (40.0%) | 14 (33.3%) | 24 (41.4%) | |
| **Peritumoral inflammation** | N/A | 84 | 18 | 15 | 51 | 0.310[1] |
| | 0 | 43 (75.4%) | 5 (62.5%) | 24 (70.6%) | 14 (93.3%) | |
| | 1 | 9 (15.8%) | 3 (37.5%) | 5 (14.7%) | 1 (6.7%) | |
| | 2 | 5 (8.8%) | 0 (0.0%) | 5 (14.7%) | 0 (0.0%) | |
| **T** | N/A | 16 | 0 | 9 | 7 | 0.227[1] |
| | 1 | 82 (65.6%) | 13 (50.0%) | 23 (57.5%) | 46 (78.0%) | |
| | 2 | 25 (20.0%) | 7 (26.9%) | 11 (27.5%) | 7 (11.9%) | |
| | 3 | 8 (6.4%) | 2 (7.7%) | 4 (10.0%) | 2 (3.4%) | |
| | 4 | 10 (8.0%) | 4 (15.4%) | 2 (5.0%) | 4 (6.8%) | |
| **N** | N/A | 43 | 0 | 8 | 35 | 0.227[1] |
| | 0 | 53 (54.1%) | 12 (46.2%) | 28 (68.3%) | 13 (41.9%) | |
| | 1 | 19 (19.4%) | 7 (26.9%) | 7 (17.1%) | 5 (16.1%) | |
| | 2 | 19 (19.4%) | 6 (23.1%) | 3 (7.3%) | 10 (32.3%) | |
| | x | 7 (7.1%) | 1 (3.8%) | 3 (7.3%) | 3 (9.7%) | |
| **M** | N/A | 84 | 10 | 27 | 47 | 0.900[1] |
| | 0 | 2 (3.5%) | 0 (0.0%) | 1 (4.5%) | 1 (5.3%) | |
| | 1 | 1 (1.8%) | 0 (0.0%) | 0 (0.0%) | 1 (5.3%) | |
| | x | 54 (94.7%) | 16 (100.0%) | 21 (95.5%) | 17 (89.5%) | |

COPD, Chronic obstructive pulmonary disease; N/A , Not available; AC, atypical carcinoid; LCNEC, large cell neuroendocrine lung cancer; SCLC, small cell lung cancer.
[1] Fisher's Exact Test for Count Data (adjusted for multiple comparisons),
[2] Kruskal-Wallis rank sum test (adjusted for multiple comparisons).

### 4.2.2. Distribution pattern of immunologic markers by tumor cells

We evaluated the differences and similarities among four immunotherapeutic markers and discovered that the expression of OX40L in AC tumor cells was significantly lower than in SCLC tumors ($p < 0.001$). In contrast, ACs exhibited substantially higher tumor cell GITR expression levels than SCLC or LCNEC tumors ($p < 0.001$). Additionally, GITR expression in SCLC tumor cells was notably higher than in LCNEC ($p = 0.011$). TIM3 expression in tumor cells was significantly higher in ACs compared to both LCNEC and SCLC tumors ($p = 0.047$ and $p < 0.001$, respectively). No significant differences were observed in VISTA expression. We performed unsupervised hierarchical clustering to determine whether LNEN subtypes could be distinguished solely based on their tumor cells' VISTA, GITR, OX40L, or TIM3 expression. The cluster analysis identified three subgroups with distinct immunologic phenotypes; however, these clusters did not correspond to the histological subtypes.

During the analysis of tumor cell marker expression, we found that grade 2 tumors generally showed higher GITR ($p = 0.028$) and TIM3 ($p = 0.03$) expression than grade 3 tumors. When examining smoking habit as a clinicopathological characteristic and GITR expression in tumor cells, it was revealed that the expression level was higher in never-smokers than in current smokers ($p = 0.046$); however, this could be explained by the individual smoking habits of LNEN patients.

### 4.2.3. Distribution pattern of immunologic markers by immune cells

We considered it important to understand the immune landscape of each LNEN subtype, and for this purpose, we examined the CD3 expression of the tumors. The abundance of immune cells was comparable in LCNEC and SCLC samples; however, it was significantly lower in LADC tumors ($p<0.001$). Additionally, ACs exhibited substantially lower levels of the immune checkpoint markers VISTA ($p<0.001$) and GITR ($p= 0.002$) compared to LCNEC and SCLC tumors. In contrast, TIM3 expression in immune cells was significantly lower in SCLC compared to both AC ($p<0.001$) and LCNEC tumors ($p<0.001$). Cluster analysis was unable to differentiate the LNEN subtypes based solely on the expression levels of VISTA, GITR, OX40L, or TIM3 in immune cells. Among the clinicopathological characteristics, we examined the relationship between the location of

the tumor within the lung and the degree of immune infiltration, which showed that centrally located tumors generally exhibited significantly lower levels of immune infiltration ($p < 0.001$), and their immune cells showed lower VISTA and TIM3 expression than peripherally located tumors ($p < 0.001$). Considering the necrotic content of the tumors, we concluded that necrotic tumors exhibited significantly higher immune infiltration than non-necrotic lesions ($p=0.027$). Tumors with high peritumoral inflammation demonstrated increased immune infiltration compared to those with medium or low levels of peritumoral inflammation.

### 4.2.4. Correlation between the expression patterns of immune-related markers defined by tumor cells and immune cells

The expression of tumor cell OX40L and TIM3 was positively correlated with VISTA expression, showing correlation coefficients of $R = 0.4928$ ($p < 0.0001$) and $R = 0.3083$ ($p = 0.0245$) (Figure 9). A positive linear correlation was observed between tumor cell TIM3 and GITR expressions, with $R = 0.4658$ ($p< 0.0001$). We also found that the expression of GITR in immune cells correlated with GITR expression in tumor cells and with OX40L expression in immune cells, yielding correlation coefficients of $R = 0.5416$ ($p = 0.0233$) and $R = 0.5678$ ($p = 0.011$), respectively.

To evaluate the impact of VISTA, OX40L, GITR, and TIM3 expressions on CD3 distribution, we correlated immune cell CD3 expression with both immune cell and tumor cell expressions of the markers above (Figure 10). None of the examined immunotherapy targets were significantly associated with CD3 expression. A trend toward a positive linear correlation was observed between immune cell GITR and VISTA expression.

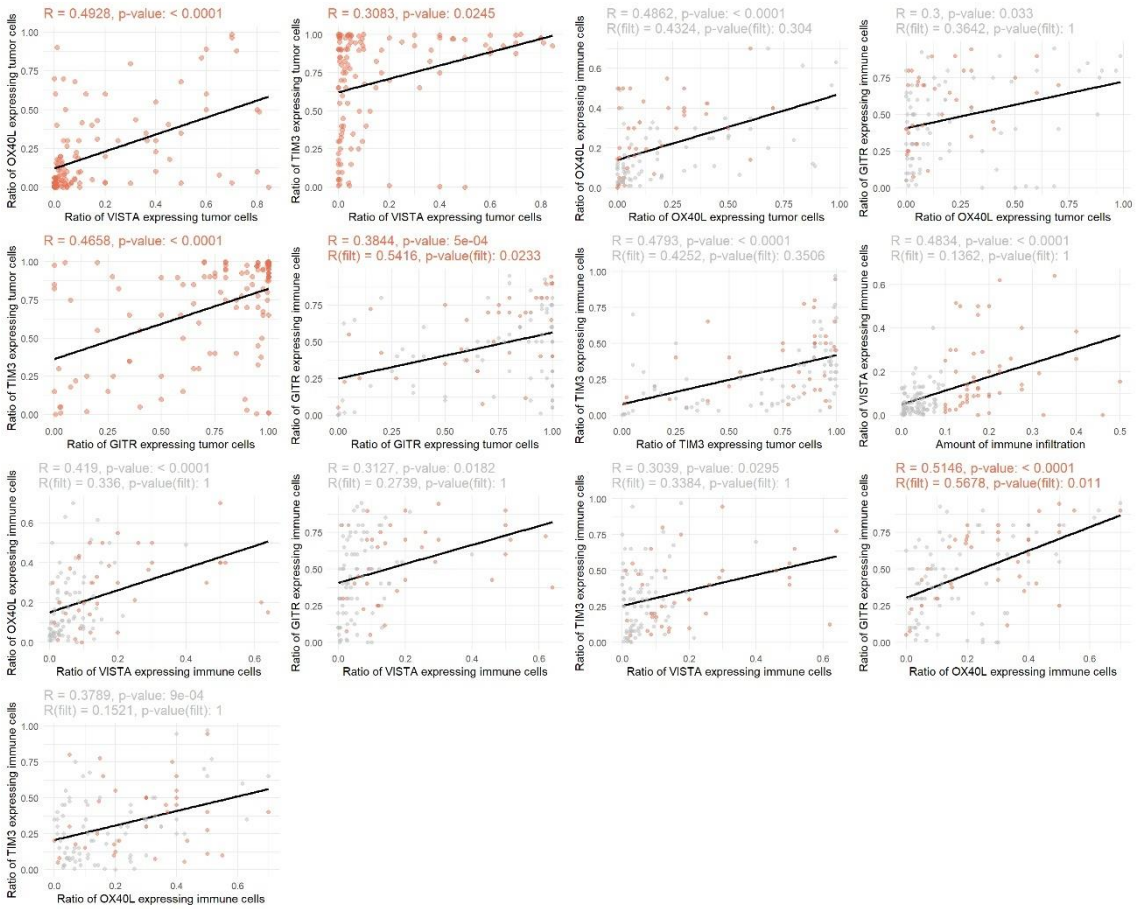


**Figure 9.** Correlation between tumor cells and immune cells, VISTA, OX40L, GITR, and TIM3 expression. Only associations that remained significant after Bonferroni correction are shown. All p-values are adjusted for multiple comparisons. Results were obtained for a filtered dataset, including samples with an immune infiltration of 10% or larger, indicated with „(filt)". If the observed correlation remained significant on the filtered dataset, the results are highlighted in orange. On panels with multiple marker colors, orange indicates samples for which immune infiltration exceeded 10%.

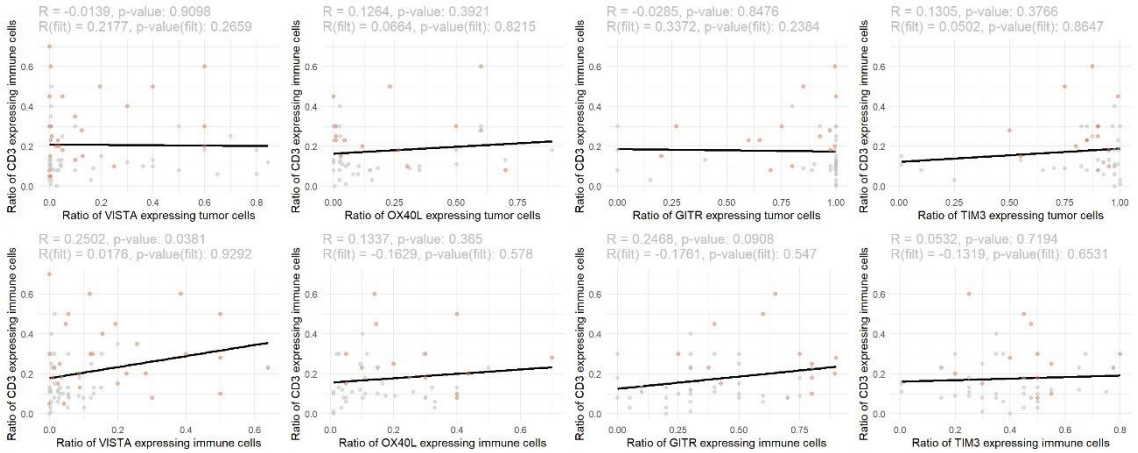


**Figure 10.** Correlation patterns between tumor cell and immune cell VISTA, OX40L, GITR, and TIM3 expression and immune cell CD3 expression in surgically resected LNENs.

### 4.2.5. LNEN subtype-specific immunological landscape examined by PC analysis

PC analysis revealed that the first three PCs account for 72% of the variance in the data. However, further investigations showed that PC1 did not effectively separate patients based on the LNEN subtype. As a result, we projected all data points and original variables into the space defined by PC2 and PC3 (see Figure 11). As shown, with the use of PC analysis, ACs can be distinguished from LCNECs and SCLCs based on their immunological and tumor cell marker expression. Specifically, we observed the following trends: (1) ACs display high levels of tumor cell markers TIM3 and GITR while showing low levels of immune cell GITR; (2) both tumor cells and immune cells of SCLCs exhibit high levels of GITR, but their immune cells have low levels of TIM3; and (3) immune cells of LCNECs show high expression levels of both GITR and TIM3.

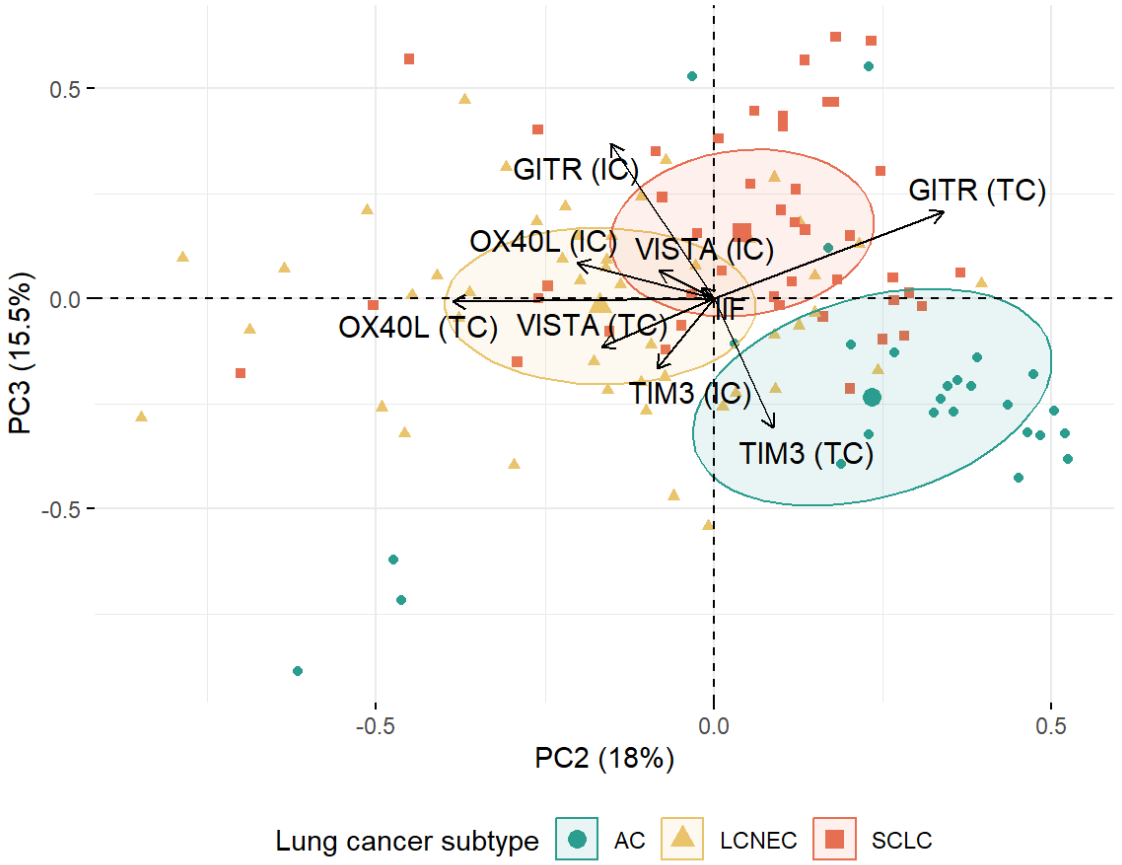


**Figure 11.** Principal component analysis of LNEN marker expression. ACs can be distinguished from LCNEC and SCLC tumors based on their immune cell and tumor cell marker expression. ACs express high tumor cell TIM3 and GITR levels and low immune cell GITR levels. Tumor cells and immune cells of SCLC lesions express high levels of GITR, and their immune cells express low levels of TIM3. Percentage values on axis labels indicate the percentage of explained variance by the principal component. Immune cells of LCNEC tumors express high levels of GITR and TIM3. AC, atypical carcinoid; LCNEC, large cell neuroendocrine lung cancer; SCLC, small cell lung cancer; and PC, principal component.

### 4.2.6. Association between immune marker expression levels and survival

Patients were categorized into low- and high expressing groups based on the median expression levels of four examined immune related markers. Although the expression levels of tumor cell markers did not significantly influence OS, patients with high tumor

cell TIM3 expression showed a tendency toward improved survival outcomes compared to those with low tumor cell TIM3 levels (p=0.08) (Figure 12/A). The expression of infiltrating immune cells did not demonstrate significant prognostic implications either. Regarding immune cell marker expression, we observed that high TIM3 expression was associated with significantly improved survival outcomes compared to low TIM3 expression (p=0.021). In contrast, low GITR expression in immune cells was correlated with an increased OS, approaching statistical significance (p=0.064) (Figure 12/B). Additionally, we conducted a multivariate Cox regression analysis to identify the clinical parameters influencing OS (Figure 13). Among all examined parameters, diabetes (p=0.003), histological type (AC vs. LCNEC, p=0.061; AC vs. SCLC, p=0.016), and tumor grade (p=0.045) were found to influence OS independently. Notably, the independent prognostic relevance of immune cell TIM3 and GITR expression remained borderline significant (p= 0.057 and p= 0.071, respectively).

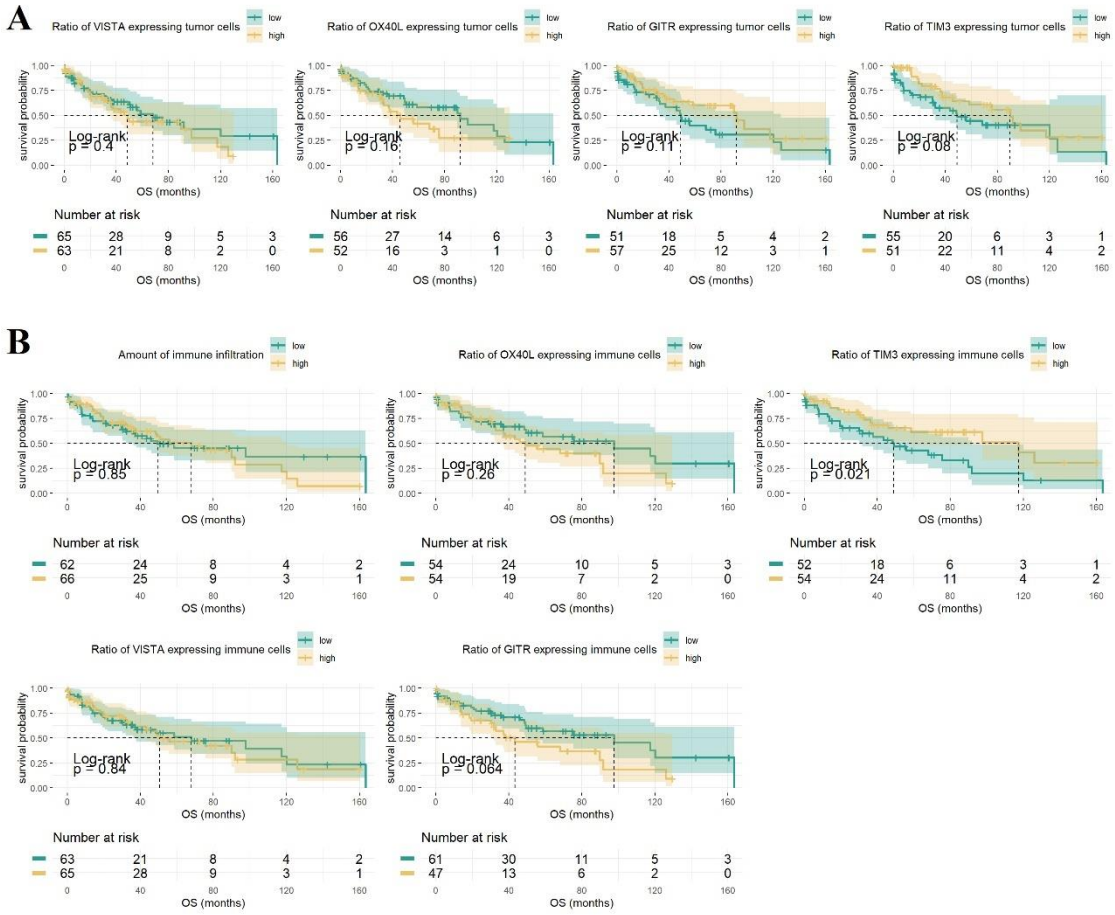


**Figure 12.** Kaplan-Meier estimates for OS concerning tumor cell **(A)** and immune cell **(B)** VISTA, OX40L, GITR, and TIM3 expression.

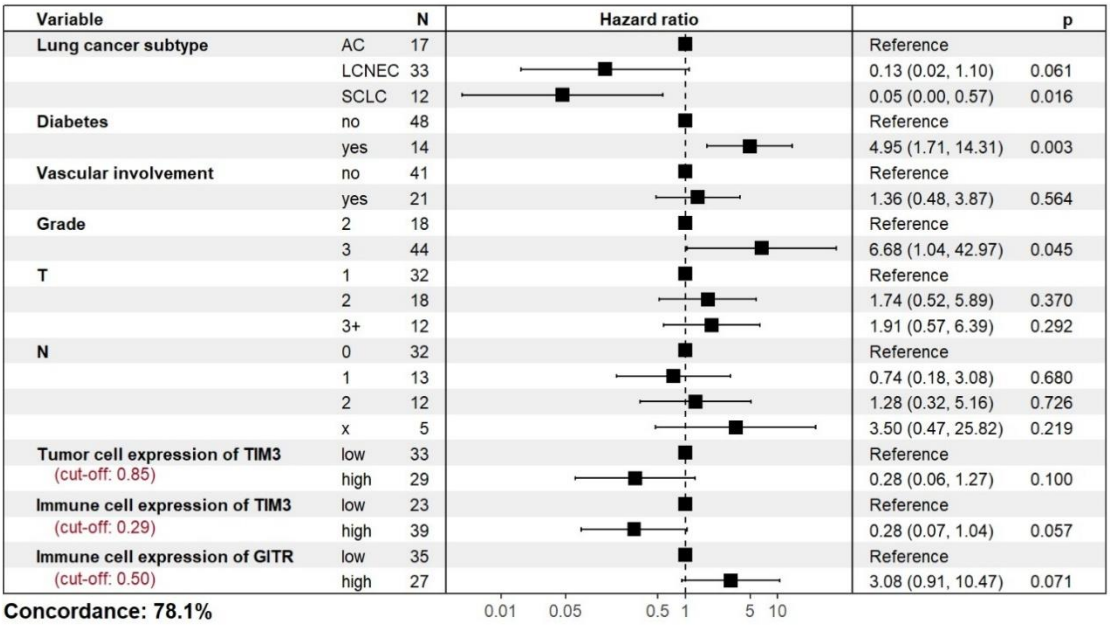


**Figure 13.** Multivariate Cox-regression model for OS. The outcomes are presented as hazard ratios (HR) and their corresponding 95% confidence intervals (CI). N indicates the number of samples belonging to a given category. P-values show the significance of the associations.

# 5. Discussion

## 5.1. KRAS- study

Based on their growth pattern, LADCs are classified by the WHO into five groups: lepidic, acinar, papillary, solid, and micropapillary (9). These distinct morphological growth patterns can occur in combination. However, the dominant pattern determines the tumor grade and clinical management (81). Lepidic adenocarcinoma is considered a low-grade (grade I) lesion, characterized as a slow-growing, non-invasive tumor, in which cancer cells proliferate along intact alveolar structures without invading surrounding tissues (82). This subtype is typically associated with early-stage disease and the best prognosis among LADC subtypes. Given their less aggressive nature, these tumors are often curable with surgical anatomical resection alone, and additional treatments such as chemotherapy and targeted therapy are usually not necessary unless the disease is in an advanced stage (83).

Acinar and papillary adenocarcinomas represent moderate-risk tumors and are classified as intermediate grade (grade II). Acinar tumors exhibit glandular structures, while the papillary tumors form finger-like projections (84, 85). These subtypes are more invasive than lepidic adenocarcinomas but less aggressive than the solid or micropapillary forms. Surgical resection remains the primary treatment, yet in case of lymph node involvement, adjuvant platinum-based chemotherapy is frequently recommended (86). Patients with EGFR or ALK mutations may also benefit from targeted therapies (87, 88).

Solid and micropapillary tumors are classified as highly invasive, high-grade (grade III) tumors and are prone to early metastatic spread, particularly to lymph nodes and distant organs (89, 90). Micropapillary adenocarcinomas consist of clusters of tumor cells floating within alveolar spaces, whereas solid adenocarcinomas are characterized by the absence of glandular differentiation, and grow in dense sheets or nests. Micropapillary LADCs are known to commonly exhibit mutations in the KRAS, EGFR, and BRAF genes (91). These patterns are linked to poor prognosis and high recurrence rates, requiring aggressive multimodal treatment. For resectable tumors, surgical excision is still performed, however, it is almost always followed by adjuvant chemotherapy to reduce the risk of recurrence. Immuno- and targeted therapies may also be applied if they are indicated based on molecular profiling. Radiation therapy might also be considered in

cases with positive surgical margins or lymph node metastases. Several studies have demonstrated that the presence of high-grade histological components is associated with a worse prognosis and reduced OS in LADCs (92, 93). Thus, early detection of these aggressive features is particularly important due to their affinity for developing therapy resistance, likelihood of relapse, and more frequent metastasis. Accurate and timely diagnosis can help establish an appropriate personalized treatment strategy.

In our patient cohort, histological heterogeneity was observed in approximately one-third of the analyzed specimens, with two cases showing more than two distinct histological components. The acinar growth pattern was the most frequently identified subtype, appearing in nearly half of the samples, followed by solid and lepidic morphologies. Solitary papillary structures were rare, accounting for only 5% of cases. The high-grade patterns, such as solid and micropapillary components, are strongly associated with higher rates of lymphovascular invasion and distant metastasis, even their minor presence within a tumor warrants careful evaluation during risk stratification and treatment planning (10, 91, 94). These results highlight the need to consider secondary aggressive components during histopathological evaluation, as their presence can significantly influence tumor progression and clinical outcome.

The relationship between KRAS mutation subtypes and tumor differentiation remains unclear. While only approximately 30% of LADCs harbor detectable KRAS mutations, their presence has been consistently associated with a poorer prognosis and reduced OS (95-97). KRAS mutations predominantly occur in codons 12, 13, and 61, leading to amino acid substitutions that influence binding affinities in signaling effector molecules and promote tumorigenic pathways. Among these, the KRAS G12C mutation, resulting from a glycine-to-cysteine transversion at codon 12, is particularly common in smoking-associated LADC (98, 99). In our study cohort, the KRAS G12C mutation was predominant, followed by KRAS G12D and KRAS G12V. In two cases, we detected two simultaneous mutations. We also identified KRAS G12D, KRAS G12S, KRAS G12R, and KRAS G12A variants. However, due to the limited sample size, rare coexisting mutations, such as KRAS G12R/ G12D or KRAS G12S/ G12A, could not be assessed. No statistically significant correlations were identified upon analysis between KRAS mutational status and histological growth patterns.

Notably, samples with lepidic morphology, which accounted for approximately one-fifth of all analyzed cases, did not contain KRAS G12A mutations. Additionally, the micropapillary growth pattern was not observed in any of the tumors with wild-type KRAS or multiple KRAS mutations. Previous reports have shown conflicting evidence regarding the existence of a significant association between KRAS mutations and LADC histology (100-102). These discrepancies underscore the need to explore molecular relationships between specific genetic mutations, dominant histological patterns, and patient prognosis to improve treatment strategies.

To more accurately map the tumor landscape and assess intratumoral heterogeneity, we analyzed three separate tumor tissue samples and one adjacent non-tumorous sample from each patient. In nineteen cases, we detected significant differences between tumor samples from the same patient regarding the dominant KRAS mutation, indicating substantial genetic variability. Interestingly, nine tumor tissue cores did not carry KRAS mutations, whereas the paired non-tumor tissue core contained a KRAS mutation. These results suggest significant variability in the distribution of KRAS mutations within and around tumors. Further research is needed to better understand the potential effects of the adjacent, non-tumorous lung tissue.

These discrepancies in mutational status across various tissue samples from the same patient raise critical questions about the reliability of diagnostic results and determining the most effective therapeutic option. In our study, we processed surgically removed tissue samples that contained the entire tumor and the adjacent surrounding tissue, allowing for a larger sample volume, thereby increasing diagnostic accuracy. By contrast, routine clinical KRAS mutation analyses most often rely on biopsy samples taken during bronchoscopy or CT-guided sampling. These smaller samples are taken from limited tumor regions and might not accurately capture the full extent of the tumor's heterogeneity. Additionally, multiple biopsy sampling is not standardized in clinical settings. Thus, resulting in less accurate findings regarding the determination of intratumoral heterogeneity (103, 104).

Patients with LADC harboring specific KRAS mutations, particularly KRAS G12C, have shown responsiveness to targeted therapies with selective inhibitors, such as sotorasib and

adagrasib. Therefore, individuals with a KRAS G12C-positive mutation are eligible for these targeted treatments (105).

In contrast, patients with other KRAS mutations, for whom targeted therapies are not yet available, remain reliant on the treatment with traditional chemotherapy (106). However, due to potential intratumoral heterogeneity, accurately defining the best diagnostic approach and identifying the optimal therapeutic strategy can be challenging.

Peritumoral inflammation varies across stages of tumor development and affects the tumor progression and invasion. Chronic inflammation can create a tumor-promoting microenvironment by activating the innate immune system and is, therefore, widely recognized as an important element of tumorigenesis (107). Tumor cells are constantly exposed to external and internal cytosolic stimuli, which can trigger acute and chronic inflammatory responses by activating several intracellular signaling pathways, including the caspase-1 inflammasome pathway (108).

Interleukin-1β (IL-1β) mediated inflammation inhibits the antitumor immune response, thus contributing to tumor growth and tumor progression (109). NLRP3 is a cytosolic multiprotein complex that detects microbial motifs, endogenous danger signals, and environmental irritants. In lung tumors, NLRP3 activation results in the release of pro-inflammatory cytokines such as IL-1β, thereby playing a prominent role in tumor-associated inflammation (110, 111). *In vitro* studies, using lung tumor cell lines, have demonstrated elevated NLRP3 expression, suggesting a potential role in modulating tumor behavior and immune interactions (112). Tengesdal et al. investigated NLRP3 expression *in vitro* and *in vivo* melanoma models, revealing that NLRP3 activation led to inflammation-mediated immunosuppression via IL-1β signaling and enhanced inflammasome production (113). Increased production of IL-1β in the peritumoral environment is known to promote the expansion of myeloid-derived suppressor cells, which leads to the inhibition of natural killer (NK) cells and CD8 T-cell activity, further contributing to the regression of the antitumor immune response (114-116).

In agreement with these findings, another study analyzing lung cancer tissue specimens and cell lines identified significant upregulation of NLRP3 gene expression in LADC (117). During histopathological analysis of LADC samples, it was observed that NLRP3 expression levels were higher in high-grade tumors, suggesting a possible association

between NLRP3 activity and a more aggressive tumor phenotype (112). Our study also revealed elevated NLRP3 expression in high-grade tumor components, particularly in solid histological patterns compared to acinar growth patterns, as assessed using a semi-quantitative evaluation scale. Given that solid histology is associated with poorer prognosis and increased tumor aggressiveness, these findings suggest that NLRP3-driven inflammasome activation may play a role in promoting more invasive and treatment-resistant tumor phenotypes (118).

Moreover, we found a positive correlation between NLRP3 expression and increased immune infiltration within the TIM. Tumors with higher NLRP3 activity expression also showed higher expression levels of the T-cell marker CD3 and macrophage marker CD163, indicating an enhanced immune system involvement in these cases. These findings are consistent with previous research that demonstrated how NLRP3 activation can recruit and modulate immune cell populations, which may contribute to immune evasion and tumor progression (119). Despite the increased expression of immune markers, the tumorous and non-tumorous tissues showed a similar trend, complicating the distinction between tumor-specific immune response and systemic inflammation. In light of this, when developing immune-targeted therapies, it should be considered that immune response modulation affects not only the tumor but also the surrounding healthy tissue.

In our studies, we found no significant correlation between increased NLRP3 expression and KRAS mutation status, suggesting that NLRP3 activation and the resulting inflammasome activity may operate independently of KRAS-driven oncogenic signaling, reinforcing that tumor-promoting inflammation can arise through multiple mutation-independent mechanisms. The literature on the relationship between the inflammatory marker NLRP3 and tumor processes remains divided, with some studies suggesting that NLRP3 promotes a pro-inflammatory microenvironment conducive to tumor progression, while others propose that it may play a role in enhancing antitumor immunity (120, 121). These results highlight a need for further research to clarify how NLRP3 influences immune regulation and tumor progression. Nevertheless, the observed correlation between elevated NLRP3 expression and increased immune cell infiltration is clinically significant. Chronic inflammation is a well-known contributor to tumor progression

(122), and our findings indicate that NLRP3 activity may play a role in recruiting and regulating the functionality of immune cell populations within the TIM.

Targeting the NLRP3 inflammasome is a promising, newly emerging therapeutic strategy. NLRP3 inhibitors, initially developed for inflammatory diseases, are now gaining interest among researchers for their potential to limit the tumor growth associated with inflammation (123). By blocking NLRP3-related inflammatory pathways, these agents may reduce the cancer-promoting effects of long-term inflammation, offering a new treatment option for inflammation-driven LADCs.

While our study provides valuable insight into the role of NLRP3 in LADC, it also has some limitations. Firstly, due to the retrospective nature of the study, clinical and follow-up data were not available for all included cases, limiting our ability to correlate the molecular findings with patient outcomes. Conducting a prospective study with longitudinal follow-up and clinical outcome assessments would improve the reliability of these observations. Furthermore, the patient cohort size is small for some results to reach statistical significance. Our hypothesis-generating outcomes presented in this study require further preclinical and clinical validation through larger studies with proper follow-up.

### 5.2. LNEN- study

The immune infiltration within the tumorous and peritumoral regions is pivotal in shaping malignant lesions' progression and the clinical outcomes of the patients (124, 125). The tumor immune microenvironment and the intricate interactions between tumor cells and the infiltrating immune cells are critical determinants of treatment response, particularly in targeted therapies and immunotherapy. Therefore, a deeper understanding of TIM dynamics is essential for developing more effective treatment protocols and optimizing patient specific therapeutic strategies (126, 127).

In this context, we explored the immunological landscape of LNENs by evaluating the expression patterns of four novel immunotherapeutic markers (VISTA, OX40L, GITR, TIM3) in surgically resected AC, LCNEC, and SCLC tumors. Given the limited availability of predictive and prognostic biomarkers in neuroendocrine neoplasms,

identifying reliable molecular markers remains a significant challenge. Orthopedia Homeobox Protein (OTP) has emerged as a potential prognostic marker for pulmonary carcinoids among the promising candidates. Furthermore, OTP and the adhesion molecule CD44 have both been proposed as biomarkers with prognostic value. However, the impact of these factors on OS and the efficacy of immunotherapy remains a subject of debate (128-130).

One of the examined biomarkers, VISTA, is a membrane protein primarily expressed by myeloid cells, granulocytes, and T cells, and functioning as an inhibitory immune checkpoint regulator for antigen-presenting cells and T cells (131). The prognostic significance of VISTA expression remains controversial; it is associated with improved OS in epithelioid mesothelioma but correlated with worse survival outcomes in colorectal tumors (132-134). In our study, VISTA expression did not significantly impact OS, but its expression was notably higher in immune cells within LCNECs and SCLCs than in adenocarcinomas. This suggests that VISTA may contribute to immune suppression in highly malignant lesions by inhibiting T cell function, thereby reducing the antitumor response. As a result, VISTA may serve as a potential immunotherapeutic target in these aggressive cancers.

Likewise, we evaluated OX40L, an immune checkpoint modulator that is primarily expressed on activated antigen-presenting cells, including dendritic cells, B cells, and macrophages (135). Its interaction with OX40, expressed on T cells, enhances CD4 and CD8 T cell survival, thereby boosting tumor-specific effector T cell responses while simultaneously counteracting the suppressive effects of regulatory T cells (Tregs) (136). A recent study on NSCLC demonstrated that elevated OX40L expression correlates with increased CD4 T cell infiltration and improved OS (137). Similar findings have been reported in SCLC, melanoma, and pancreatic ductal adenocarcinoma, highlighting its potential prognostic and therapeutic significance (138, 139). In our study, OX40L expression was not significantly associated with survival outcomes, which may, in part, be attributed to the limited sample size. However, we observed that AC tumor cells exhibited lower OX40L expression than other LNEN subtypes. Interestingly, in our working group's previous study on LNENs, we did not detect significant differences in OX40 expression across histological subtypes, suggesting that OX40 and OX40L may exhibit independent expression patterns (140).

GITR is a costimulatory receptor expressed on T cells and NK cells, playing a crucial role in effector T cell activation (141). Given its ability to enhance anti-tumor immunity, GITR is a promising immunotherapeutic target. Preclinical models show that GITR activation boosts T cell responses while inhibiting Treg function, and several clinical trials are currently evaluating GITR-targeted therapies (142, 143). In our study, GITR expression varied by LNEN subtype. AC tumor cells showed higher GITR levels than LCNEC and SCLC tumor cells. In contrast, immune cells within ACs exhibited significantly lower GITR levels than those in LCNEC and SCLC tumors. This observation suggests that the tumor histology type and the immune cell infiltration patterns may influence GITR expression. Furthermore, the effects of GITR on tumor-infiltrating immune cells appear to be time dependent. Initially, GITR activation suppresses Treg function, promoting immune infiltration. However, prolonged GITR stimulation may ultimately suppress antitumor immunity (144-148). Our research group's previous study further supported this, showing that AC tumors, despite high GITR expression, had fewer CD8 and CD3 tumor-infiltrating lymphocytes than LCNEC and SCLC tumors, where GITR expression was highest (140). From a prognostic perspective, our analysis found that low GITR expression in the TIM was associated with a borderline significant trend toward improved survival. This pattern remained consistent in Cox regression models. These findings highlight the complex role of GITR in tumor immunity and its potential as a therapeutic target in LNENs.

Lastly, TIM3 is a negative immune checkpoint regulator primarily expressed on natural killer cells and macrophages. While unbound TIM3 enhances T cell activation, its engagement upon activation leads to immune suppression, inhibition of anti-tumor immune responses, and poorer prognosis (149-151). Preclinical studies have demonstrated that dual blockade of TIM3 and PD-1 induces tumor regression, and several clinical trials are currently investigating TIM3-targeted therapies in solid tumors (149, 150, 152, 153). In our study, TIM3 expression was higher in both tumor cells and immune cells of ACs compared to LCNEC and SCLC tumors. From a clinical perspective, elevated TIM3 expression in both tumor cells and immune cells was associated with a trend toward improved survival. TIM3 expression did not emerge as an independent prognostic factor in our multivariate analysis. Despite this, the high TIM3 expression in AC tumors suggests that it may serve as a potential subtype-specific immunotherapeutic

target. Further investigations are required to determine whether TIM3 blockade could enhance immune responsiveness in AC patients and improve immunotherapy efficacy in LNENs.

LNENs exhibit highly diverse immune phenotypes, with the expression of VISTA, OX40L, GITR, and TIM3 playing a critical role in modulating the tumor immune microenvironment through complex and time-dependent regulatory mechanisms (144, 146, 147, 149-151, 153-158). These dynamic interactions should be carefully considered when evaluating their impact on intratumoral immune cell distribution and antitumor immune responses. Despite the insights gained from this study, several limitations must be acknowledged. A small subset (4.9%) of surgically resected tissue samples was older than 15 years, which may have affected sample integrity and biomarker stability. Additionally, due to the study's retrospective nature, clinical and follow-up data were unavailable for all cases, limiting our ability to draw definitive conclusions regarding prognostic implications. Although our study comprises a relatively large collection of rare tumors, the cohort size remained insufficient to achieve statistical significance in specific analyses. Furthermore, the study is mainly descriptive and hypothesis-generating rather than confirmatory, as the direct effects of immunotherapeutic agents on LNEN immune modulation could not be assessed.

## 6. Conclusions

The aim of our first study was to characterize the role of the NLRP3 inflammasome in KRAS mutant LADCs. We attempted to map the relationship between the TIM and inflammatory signaling pathways. We observed significant differences in clinical behavior among the five subtypes of LADCs. Concerning the KRAS mutations, G12C was the most common in our cohort, followed by G12D and G12V. We compared different subtypes of LADC based on KRAS mutations, which showed significant intratumor variability. In nineteen KRAS G12C mutant patients, different tissue samples from the same tumor harbored distinct mutational profiles, indicating that there is a high level of genetic heterogeneity within the tumor.

Our results revealed a significant relationship between NLRP3 expression and immune cell infiltration. Increased NLRP3 levels were associated with enhanced immune responses, and thus, part of the tumor's immune system. Additionally, we analyzed the relationship between NLRP3 levels and specific KRAS mutant variants. However, we did not find a statistically significant correlation between them. Thus, it is likely that inflammatory pathways are formed independently of KRAS-associated tumor development. These findings underscore the potential of targeting the NLRP3 inflammasome as a novel therapeutic strategy for patients with LADC.

Our second study provided valuable insights into the immunophenotypic diversity of LNENs. We identified distinct immunological profiles by examining the expression patterns of four novel immunotherapeutic markers, namely VISTA, OX40L, GITR, and TIM3, across different LNEN subtypes. TIM3 expression was significantly elevated in AC, while GITR expression was most pronounced in SCLC and LCNEC. Immune cell infiltration differed among LNEN subtypes. Lower levels of immune markers observed in AC may indicate that their tumor immune environment is more immunosuppressive. Survival outcomes were better in the case of TIM3 expression, suggesting that TIM3 may be a potential prognostic biomarker in LNENs. Together, these results highlight the need for a subtype-specific immunotherapeutic approach and further investigation into the efficacy of immune checkpoint inhibitors targeting TIM3, GITR, and VISTA.

## 7. Summary

Our work emphasizes the complexity of LADC and LNENs. It highlights the importance of histological diversity, genetic variability, inflammation, and the dynamics of the immune microenvironment. The initial study focused on the heterogeneity of LADC, examining the KRAS mutation status, its relationship with histopathological growth patterns, and the role of NLRP3 inflammasome activation in the immune response.

In our analysis, 19 cases exhibited significant intratumoral heterogeneity in KRAS mutations, indicating genetic variability within the tumor. According to the growth pattern, it can be said that KRAS G12C mutations were present in lepidic LADC, while wild-type KRAS gene mutations were absent in micropapillary LADC. Nevertheless, we did not find a significant correlation between KRAS mutations, histological growth patterns, and mucin secretion. Beyond histological and genetic analysis, we explored the expression of the inflammatory factor NLRP3 and its relationship with immune cell infiltration and the TIM. Our findings indicate that high NLRP3 expression is associated with an inflammatory microenvironment and increased immune cell infiltration, yet no significant correlation was observed between NLRP3 expression and KRAS mutational status.

In the second part of our research, we analyzed the expression of four immunotherapeutic markers (VISTA, OX40L, GITR, TIM3) in LNENs to gain deeper insights into the tumor immune microenvironment and potential therapeutic targets. Our findings reveal distinct patterns of immune checkpoint expression across LNEN subtypes, with ACs exhibiting high TIM3 expression in tumor cells. In contrast, we observed an increase in GITR expression in ACs and SCLCs. In the case of OX40L, the highest values were observed in SCLCs. In ACs, as intermediate-grade malignant tumors, the expression levels of OX40L, VISTA, and GITR were also significantly lower. All these results may indicate significant differences in immune regulation between specific subtypes. These extensive results contribute to a better understanding of immune modulation in pulmonary malignancies and provide a foundation for future targeted immunotherapeutic strategies.

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## 9. Bibliography of the candidate's publications

### 9.1. List of publications that served as a basis for the current thesis

1. **K. Torok**, B. Ferencz, K. Boettiger, M. D. Pozonec, O. Pipek, J. Bogos, A. Lantos, Z. Hegedus, K. Schelch, P. Radeczky, K. Bogos, V. Teglas, E. Megyesfalvi, A. Ferenczy, F. Renyi-Vamos, C. Aigner, Z. Megyesfalvi, B. Dome, and J. Fillinger, "Impact of KRAS mutation subtypes on morphological heterogeneity and immune landscape in surgically treated lung adenocarcinoma," Translational Lung Cancer, vol. 14, no. 6, pp. 1914–1928, 2025. **IF: 3,5**

2. Ferencz B, **Török K**, Pipek O, Fillinger J, Csende K, Lantos A, Čserneková R, Mitták M, Škarda J, Delongová P, Megyesfalvi E, Schelch K, Lang C, Solta A, Boettiger K, Brcic L, Lindenmann J, Rényi-Vámos F, Aigner C, Berta J, Megyesfalvi Z & Döme B. Expression patterns of novel immunotherapy targets in the intermediate and high-grade lung neuroendocrine neoplasms Cancer Immunology, Immunotherapy 2024 May 2;73(6):114. doi: 10.1007/s00262-024- 03704-7. **IF: 5,1**

## 9.2. Other publications

**2025.**

1. Csaba M, Ghimessy ÁK, Radeczky P, Megyesfalvi Z, Kocsis Á, Agócs L, Döme B, Fehér C, **Török K**, Mészáros L, Bogyó L, Gieszer B, Csende K, Nagy D, Tihanyi H, Tarsoly G, Lality S, Hartyánszky KI, Kass J, Vágvölgyi A, Lungu V, Szegedi R, Yu E, Gyenge B, Afari D, Köllő A, Madurka I, Rényi-Vámos F. Három intézet összefogása a központi régió magas színvonalú mellkassebészeti ellátásának érdekében [The cooperation between three institutions in favor of high-standard thoracic surgical care in the central region of Hungary]. Orv Hetil. 2025 Feb 9;166(6):203-209. Hungarian. doi: 10.1556/650.2025.33240. PMID: 39923214. **IF: 0,9**

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2. Ghimessy Á, Radeczky P, **Török K**, Bogyó L, Csende K, Mészáros L, Gieszer B, Tihanyi H, Tarsoly G, Csaba M, Lality S, Hartyánszky KI, Kocsis Á, Madurka I, Agócs L, Rényi-Vámos F. Robotasszisztált műtétek helye a mellkassebészetben. Saját tapasztalatok [Robot-assisted thoracic surgery. Our first experiences]. Magyar Onkológia 2024 Sep 19;68(3):223-228. Hungarian. Epub 2024 Jul 30. PMID: 39299688. **IF: 0**

3. Bogyó LZ, **Török K**, Illés Z, Szilvási A, Székely B, Bohács A, Pipek O, Madurka I, Megyesfalvi Z, Rényi-Vámos F, Döme B, Bogos K, Gieszer B, Bakos E. Pseudomonas aeruginosa infection correlates with high MFI donor-specific antibody development following lung transplantation with consequential graft loss and shortened CLAD-free survival. Respir Res. 2024 Jul 1;25(1):262. doi: 10.1186/s12931-024-02868-1. PMID: 38951782; PMCID: PMC11218249. **IF: 5,0**

4. Á. Ghimessy, P. Radeczky, Á. Kocsis, L. Agócs, **K. Török,** K. Csende, H. Tihanyi, G. Tarsoly, M. Csaba, S. Lality, I. K. Hartyánszky, and F. Rényi-

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