Dissertation

The influence of various factors on the survival rate of mucinous and non-mucinous lung adenocarcinoma

submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

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Disclosure

Parts of this thesis were published in October 2015 in the European Journal of Pathology (Virchows Achiv): Architectural and immunohistochemical patterns of mucinous pulmonary adenocarcinomas, their relationship to *KRAS* mutation, prognosis and survival.

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Contents

Abbreviations and Definitions

AAH	Atypical adenomatous hyperplasia
LUAD	Lung adenocarcinoma
AIS	Adenocarcinoma in situ
ALK	Anaplastic Lymphoma Kinase
ATS	American Thoracic Society
BAC	Bronchioloalveolar carcinoma
CK	Cytokeratin
CTA	CT angiography
EGFR	Epidermal growth factor receptor
ERS	European Respiratory Society
HC	Histochemistry
HRCT	High-resolution Computer tomography
IASLC	International Association for the study of Lung Cancer
IHC	Immunohistochemistry
IMA	Invasive mucinous lung adenocarcinoma
KRAS	Kirsten rat sarcoma
LCLC	Large Cell Lung Carcinoma
LN	Lymph nodes
LSCC	Lung Squamous Cell Carcinoma
MRI	Magnetic resonance imaging system
NSCLC	Non-small cell lung cancer
SCLC	Small cell lung cancer
TKI	Tyrosin-kinase-inhibitor
TNM	Tumour Nodes Metastasis classification
TTF1	Throid Transcription Factor-1
WHO	World Health Organisation

Abstract in German

Das Adenokarzinom der Lunge ist die häufigste Art von Lungenkrebs und eine der gefährlichsten Krankheiten der Menschheit, die bei Nicht-Behandlung zum Tode führen würde. Es macht etwa 40-50 % aller Lungenkarzinome aus, was 50-60 % aller NSCLC-Fälle entspricht. Etwa ein Drittel dieses Spektrums sind muzinöse Adenokarzinome. In der Literatur wird berichtet, dass die muzinösen Adenokarzinome im Vergleich zu den Nicht-muzinösen einen schlechteren Outcome haben. Invasiv muzinöse, kolloide und enterische Adenokarzinome sind Varianten der Adenokarzinome. Wir haben 335 chirurgisch behandelte Adenokarzinome zwischen den Jahren 2002 bis 2012 identifiziert. Von diesen 335 Fällen untersuchten wir die 76 invasiv muzinösen Adenokarzinome, einschließlich der Kolloidvarianten, für ihre vorherrschenden und sekundären Muster, ihre unterschiedliche Form der Muzinspeicherung und -freisetzung, der Expression von *Cytokeratin 7* und *20*, *TTF1* und *CDX2*, *MUC1*, *MUC2* und *MUC5AC* Proteine, *p14* und *p16* Proteine, ihre möglichen Rearrangements für *EML4ALK* und *ROS1* sowie den *KRAS*-Mutationsstatus. Wir korrelierten diese mit dem Überleben und verglichen sie mit 259 nicht-muzinösen Adenokarzinomen.

Die klinischen Daten von Patienten mit muzinösen und nicht-muzinösen Adenokarzinomen der Lunge wurden retrospektiv gesammelt. Weiters wurden folgende Daten unter Berücksichtigung von Parametern wie Geschlecht, Raucher/Nichtraucher, Packungsjahre, postoperative Behandlungen, Chemotherapie, Strahlentherapie, Metastasenprofil und der Grund für den Tod des/der Patienten/-in als Folge des Tumors erhoben und verglichen.

Obwohl allgemein angenommen wird, dass die muzinösen Adenokarzinome sich aggressiver verhalten als nicht-muzinöse, wurden zytomorphologische und architektonische Merkmale nicht ausgewertet und nicht mit der Prognose und genetischen Aberrationen korreliert. Diese Studie beabsichtigt diese Fragen in einer Reihe von 76 Fällen von einer einzigen Institution zu beantworten.

Es ergab sich ein statistisch signifikanter Unterschied zwischen T- (log rank test ; P= 0.00067) und N-Stadien (log rank test ; P= 0.000073). Die korrespondierende Hazard Ratio der muzinösen Karzinome war 1.31 (95%CI: 0.77 bis 2.20). Das relative Risiko von 1 wäre neutral. Das Gesamtüberleben für invasiv muzinöse Adenokarzinome, die

anhand der T- und N-Stufe eingeteilt wurden, war nicht anders als ihr nicht-muzinöses Gegenstück. Die meisten waren mit einem azinären Muster gekennzeichnet. Weder das Muster noch die Art der Muzinspeicherung und -freisetzung wie z. B. luminaler, extrazellulärer oder Becherzell-Typ, hatten einen Einfluss auf das Überleben.

Nach Einteilung in klinischen Gruppen gab es keinen Unterschied zwischen muzinösen und nicht-muzinösen Adenokarzinome der Lunge in Bezug auf das Überleben, wobei das Konfidenzintervall jetzt 0,63 bis 1,83 war. Das widerspricht der Literatur, die besagt, dass die muzinösen eine schlechtere Prognose haben als nicht-muzinöse Tumore.

Von den Adenokarzinomen, die *CK20* exprimieren, exprimierten alle *TTF1* entweder stark oder zumindest fokal mit Ausnahme von einem, 8 co-exprimierten *CDX2* fokal. Die meisten muzinösen Adenokarzinome exprimierten entweder *MUC1*- oder *MUC5AC*-Proteine, aber selten *MUC2*, während in wenigen Fällen beide oder alle drei exprimiert wurden. Die negative *p16*-Expression korrelierte mit einem schlechteren Ergebnis. Muzinöse Adenokarzinome weisen in 56% der Fälle eine Mutation des *KRAS*-Onkogens auf. Der *KRAS*-Mutationsstatus korrelierte weder mit dem Architekturmuster noch mit dem Überleben der Patienten. Am häufigsten traten Mutationen in Codon 12 auf; ein Fall wies zudem doppelte *KRAS*-Mutationen in den Codons 12 und 61 auf. Die Becherzell-Varianten der muzinösen Adenokarzinome präsentierten sich überwiegend mit Codon-12-Mutationen, während alle Kolloidvarianten eine *KRAS*-Mutation hatten. Zwei Fälle hatten EML4- und ALK1-Rearrangements; *ROS1* Rearrangement konnte nicht gefunden werden. Muzinöse Adenokarzinome verhalten sich ähnlich wie nicht-muzinöse Varianten. Der wichtigste Faktor, der das Gesamtüberleben voraussagt ist das TNM-Stadium, gefolgt von negativer *p16*-Expression.

Abstract in English

Lung Adenocarcinoma is the most common type of lung cancer and one of the most dangerous diseases of humankind, which can lead to death if not treated. It accounts for approximately 40-50% of all lung cancers, representing 50-60% of all NSCLC cases. Within this spectrum, approximately one-third differentiate along a mucinous pathway. It has been claimed, that mucinous adenocarcinomas behave worse than their non-mucinous counterpart. Invasive mucinous, colloid, and enteric adenocarcinomas are variants of adenocarcinomas. Between 2002 and 2012 we identified 335 surgically resected cases of mucinous and non-mucinous adenocarcinomas. The aim of the study is to compare the overall survival as well as the progression-free survival of mucinous versus non-mucinous adenocarcinomas of the lung. We also investigated 76 mucinous adenocarcinomas, including colloid adenocarcinomas, for their predominant and secondary patterns, their different form of mucin storage and release, their expression of *cytokeratins 7* and *20*, *TTF1* and *CDX2*, and *MUC1*, *2* and *5AC* proteins, their expression of *p14*, and *p16* proteins, their possible rearrangements for *EML4ALK* and *ROS1*, as well as their *KRAS* mutational status and correlated this with survival. For comparison 259 non-mucinous adenocarcinomas were selected.

The clinical data of Patients with mucinous and non-mucinous adenocarcinoma of the lung were collected retrospectively. The following data also have to be compared considering parameters such as gender, smoking / non-smoking, pack-years, postoperative treatments, chemotherapy, radiotherapy, metastatic profile, and the reason for the death of the patient as a result of the tumour (cancer-related death).

Although it is an accepted assumption, that mucinous LUADs if stratified by stage behave more aggressively than their non-mucinous counterparts, cytomorphological and architectural features have not been evaluated and correlated with prognosis and genetic aberrations. Therefore we aim to address these features in 76 cases derived from a single institution.

There was a statistically significant difference between T- (log-rank test; P=0.00067) and N-stages (log-rank test; P=0.000073). The corresponding hazard ratio of mucinous carcinoma was 1.31 (95%CI: 0.77 to 2.20). The relative risk of 1 would be neutral. Overall survival of mucinous adenocarcinomas corrected for T and N stage was not

different from their non-mucinous counterpart. The acinar pattern was most frequently seen. Neither pattern, nor type of mucin storage, such as luminal, extracellular, or goblet cell type had any influence on survival.

There was no difference between mucinous and non-mucinous adenocarcinomas of the lung concerning to survival after adjusting for clinical groups, however, the confidence interval was now 0.63 to 1.83. This contrasts the literature stating that the mucinous have a worse prognosis than the non-mucinous tumours. Several adenocarcinomas were positive for *CK20*, all of them except one expressed *TTF1* either strongly or at least focally, and eight coexpressed *CDX2* focally. Most mucinous adenocarcinomas expressed either *MUC1* or *MUC5AC* proteins, rarely *MUC2*. Some cases coexpressed both or all three. In mucinous adenocarcinomas, a loss of *p16* expression correlated with a worse outcome. Mucinous adenocarcinomas exhibit mutation of *KRAS* oncogene in 56%. *KRAS* mutational status was neither correlated with architectural pattern nor survival. Most frequent were codon 12 mutations, one case presented with double *KRAS* mutations in codon 12 and 61. Goblet cell variants of mucinous adenocarcinomas presented predominantly with codon 12 mutations. All colloid adenocarcinomas had *KRAS* mutation. Two cases had EML4 and ALK1 rearranged, *ROS1* rearrangement was not found. Mucinous adenocarcinomas behave similarly to non-mucinous variants. TNM stage is the most important factor followed by *p16* loss predicting overall survival.

1 Introduction

Lung cancer is the most common cancer in men worldwide and the number one cancer leading to death in developed and developing countries.[1][2][3][4][5] Worldwide there are more than a million lung cancer-related deaths due to late detection of the primary tumour and early metastasis.[6][7][8][9][10] According to GLOBOCAN 2018, lung cancer accounts for 11.6% of all newly diagnosed cancer cases and 18.4% of all cancer-related deaths in the world.[11] Compared to the data from 2008 there is a significant decrease in newly diagnosed cancer cases (17%) and in cancer related deaths (23%) concerning lung cancer.[4] Malignant lung tumours cause 25% of cancer-related deaths.[12] The incidence of lung cancer mortality among women is increasing, because of the increasing smoking behavior of women.[13][14] The 5-year survival of lung cancer is almost 15%.[15][16][13] The tumour is also the cause of huge economic costs.[17]

Lung cancer is very heterogeneous with at least 50 different histological, biological, genetic, clinical responses very different to treatment.[18]

There are two main categories of lung cancer: small-cell lung cancer (SCLC) accounts for approximately 15% of cases, while non-small-cell lung cancer (NSCLC) makes up the remaining 85% of cases. Within the category of non-small-cell lung cancer (NSCLC), there exist three subtypes: adenocarcinoma (LUAD), squamous-cell carcinoma (LSCC), and large-cell carcinoma (LCLC).[19] Lung adenocarcinoma is the most common[20] and one the most dangerous types of lung cancer, which will lead to death if not treated. It has typical histomorphological changes and constitutes 40-50% of all lung carcinomas and represents 50-60% of all NSCLC cases.[21][22][5][23][24][25][26][19][27][28] Within this spectrum, approximately one-third differentiate along a mucinous pathway. Mucinous differentiation is already present in the precursor lesions and the origin is basal or peripheral stem cells with the capability to produce different types of mucin.[29][30]

Pulmonary adenocarcinomas usually occur in peripheral lung tissue.[31][32] The prognosis depends on lymph node involvement, the presence of vascular invasion, and

the tumour stage. The treatment depends on tumour histology, extent, age, pulmonary function, and comorbidity. It has been proposed, that mucinous adenocarcinomas behave worse than their non-mucinous counterpart. The histological subtypes namely adenocarcinoma in situ, minimally invasive adenocarcinoma, and lepidic-predominant adenocarcinoma have a 5-year survival approaching 100%, whereas micropapillary-predominant and solid with mucin-predominant adenocarcinomas are associated with particularly poor survival. Papillary-predominant and acinar-predominant adenocarcinomas have an intermediate prognosis. This effect persists also according to the stage.[33][20]

The study aims to detect the influence of various factors for example smoking behavior, therapy, histological subtypes, genetic changes, different biomarkers, etc. on the survival rate of the mucinous and non-mucinous adenocarcinoma of the lung. It has never been clearly demonstrated, however, it is always predicted that survival is worse in mucinous adenocarcinomas. So it is generally assumed that when stratified by stage, mucinous LUADs behave more aggressively than non-mucinous LUAD, cytomorphological and architectural features have not been evaluated and correlated with prognosis and genetic aberrations.[34][35][33] This study intends to address these issues in a series of 76 cases from a single institution.

2 Theoretical Background of Lung Adenocarcinoma

2.1 Definition

Lung adenocarcinoma is a primary lung cancer, which shows typical histomorphological changes and because of slow growth usually becomes invasive after years. It is the most common type of lung cancer regardless of whether the patient is a smoker or non-smoker. In the past few years, it has replaced lung squamous cell carcinoma (LSCC) of the lung in frequency and became the most common type of lung cancer despite the smoking status. The incidence has increased in the last few decades in western countries. Approximately 40-50% of all lung cancers comprise adenocarcinomas, representing 50-60% of all NSCLC cases.[21][22][5][23][24][25][26] It is the most common lung cancer of never-smokers.[36]
Squamous cell carcinoma follows, along with other non-small cell lung cancers, small cell lung cancer, and others. Among all types, including adenocarcinoma, the majority of cases occur in smokers. However, the proportion of non-smokers among adenocarcinoma patients is the highest, accounting for about one-third of all cases. In all other types, only a very small proportion of cases occur in non-smokers. About 98% of lung cancers are carcinoma with epithelial characteristics, the remaining group are sarcomas or hematopoietic and germ cell tumours.[37][38]

The tumour usually occurs in peripheral lung scar tissue such as in an earlier tuberculosis. It usually spreads in an early stage through lymphogenic way to lymph nodes around and mediastinal as well through haematogenic way specifically to the liver, bones, adrenal cortex, and brain. Lung adenocarcinomas are small in mass, but they metastase in an early stage. It is also a slowly growing cancer and could take years until it becomes invasive. It can also spread locally through intrapulmonary metastases.

There are more than 50 lung tumour types, which are documented by the World Health Organization. The following tumours are the most common forms of them,

which are the 8 most important major groups of lung cancer: Squamous cell carcinoma, small cell carcinoma, adenocarcinoma, large cell carcinoma, adenosquamous carcinoma, sarcomatoid carcinoma, carcinoid tumour, salivary gland-like carcinoma. (WHO)

Lung adenocarcinoma has an increased incidence in smokers, but this cancer type is also the lung cancer type, which occurs most commonly in non-smokers and also in women and people younger than 45.[26] The studies about the different forms of cancer are of value when considering the best therapy for each form of cancer type. Lung Squamous Cell Carcinoma (LSCC) accounts for 35% of malignant lung tumours and, in contrast to adenocarcinoma, has been decreasing in incidence in the last years.

Approximately one-third of lung adenocarcinomas are mucinous, including the colloid type, which are characterized by abundant mucin production both intracellularly and extracellularly. Mucinous differentiation can be visualized through positivity for mucicarmine as well as through Alcian blue staining of at least 80% of tumour cells. In the colloid type, there are large pools of mucin with few solid tumour nests, giving the tumour a gelatinous or colloidal appearance.

2.2 Symptoms

Lung adenocarcinoma has most commonly(70% of the time) a late onset of clinical symptoms, most often characterized by hemoptysis due to tumour cell necrosis and vessel erosions.[18] Some of the early symptoms are chronic cough with fatigue, weight loss, chest pain, and wheezing followed by difficulty in breathing. Dyspnoea usually occurs as a late-stage manifestation.[39][40]

Pleural effusions associated with lung adenocarcinoma can result from pleuritis due to pneumonia retention or from tumour infiltration of the pleura. Tumour cells in pleural effusion are a sign of worse prognosis, especially for lung adenocarcinoma and small cell lung carcinoma. Pronounced mediastinal tumours manifest frequently through superior vena cava syndrome, very often in small cell lung cancer with mediastinal lymph node metastasis. The first symptoms of primary adenocarcinoma are very common due

to brain metastases. The different clinical courses are probably because of the high heterogeneity of lung tumours.[18]

Lung adenocarcinomas usually occur in the outer parts of the lung tissue and could be present for a long time before diagnosis. Well-known symptoms of lung cancer such as chronic cough and hemoptysis may only occur at later stages of the disease. Early symptoms, that may be overlooked include fatigue, mild shortness of breath, back, shoulder, or chest pain, dysphagia, hoarseness, poor appetite, and weight loss.

If the tumour has already spread to other parts of the body, it can cause symptoms such as bone pain in case the tumour has spread to the bones. Liver dysfunction can occur if the tumour has spread to the liver. If the tumour has spread to the brain symptoms such as headache, vertigo, character change or vision disorders can be expected.

2.3 Etiology

The exact cause of lung cancer, like that of most other cancers, is not well understood; however, there are many risk factors correlated with cancer formation.

More than 90% of malignant lung tumours and even 95% of all bronchial carcinomas are caused by smoking.[18][39] There are about 7000 chemicals in tobacco and several of them are cancerogenic in experimental studies, such as polycyclic hydrocarbons of the type of benzo-(A)-pyrene, cancerogenic metal compounds (nickel carbonyl, cadmium hydroxide), N-nitroso compounds, aromatic amins and other.[18] In the USA smoking is the leading cause of all cancer-related deaths with 30%. There is a proven association between smoking and the occurrence of lung adenocarcinoma. It is reported that patients with adenocarcinoma of the lung in clinical stage 1A, who have a heavy smoking history are associated with poor outcomes and statistically significant tumour invasion.[41] The incidence of lung carcinoma death is decreasing amongst men and increasing amongst women along with the incidence of adenocarcinomas of the lung, due to the increased smoking behavior of women. Smoking-related risk of lung cancer

decreases over time after the cessation of smoking. Lung adenocarcinomas usually occur in the peripheral lung, maybe because of the increasing use of filters in cigarettes preventing the entrance of larger particles.[36][26][42]

The secondary risk factors for lung cancer are age, familial clustering, exposure to secondhand smoke, mineral and metal dust, asbestos, or radon.[43] The risk of getting lung adenocarcinoma is increased by smoking, breathing tobacco smoke from cigarette, cigar, and pipe smoking, if one is exposed to radon gas, asbestos, or other cancer-causing agents such as uranium, arsenic, vinyl chloride, nickel chromates, coal products, chloromethyl ethers, gasoline, and diesel exhaust.

There was a significant increase in cigarette consumption of the population in Germany between 1960 to the middle of 1990. Interestingly there was also in correlation an increase of lung tumour deaths within the same period. There was an increase of smokers among women and the number of smokers among men decreased. This was also reflected in the incidence of lung tumours in men and women. The incidence of lung tumours in non-smokers is 3.4 per 100,000 population per year, and it rises to 51 if someone smokes 10 cigarettes a day, if someone smokes 40 cigarettes a day to 217.5 per 100,000 per year.[18]

There are many other risk factors such as age, familial clustering, and exposure to secondhand smoke, job-related risk factors, exposure to mineral and metal dust for example chrome, nickel, arsenic, wood dust, coke oven gas, radiation for example in uranium mining, asbestos, radon.[18] Air pollution as a carcinogen has less importance in locations where air pollution is a problem.[18]. If someone has a first-degree family member with adenocarcinoma of the lung, it doubles the risk of getting lung cancer.[44]

Lung adenocarcinoma is the most common form of lung cancer in women and people under the age of 45. Nonetheless, it is often found in non-smokers and it is more common in Asia.[45]

2.4 Diagnosis

The Investigation of a patient, presenting with pulmonary symptoms, or diagnosed to have a mass in the lung, should be initiated by collecting patient history including smoking habits and whether he or she is living with a smoker or is exposed to asbestos or other cancer-causing agents. Thereafter a chest X-ray and eventually an ultrasound examination are performed. If you find a tumour bulk such as enlarged lymph nodes or tumour bulk in the chest you have to perform a HRCT (High-resolution Computer tomography) scan of the thorax. HRCT scan allows you to determine the nature, position, and extent of the mass. HRCT can also be used to get biopsy samples of the tumour to ensure the diagnosis. For the diagnosis, the tissue must be analyzed microscopically. Through the means of a bronchoscopy, a CT, or an ultrasound-guided biopsy tumour samples can be won.[46][22]

You can also perform a thoracocentesis if there is fluid in the chest to get samples of the fluid. The biopsy materials and the sample of the accumulated fluid in the chest or sputum samples have to be sent to the pathologist to review the results of the biopsy and sputum samples to confirm the diagnosis of cancer. Further, it is important to examine the samples of sputum for infectious reasons. The PET scan is important for looking at the function of the tissue rather than the tomography. Lung tumours show intense metabolic activity on a PET scan, and because of that some centers offer combined PET-CT-scan so that metastases can be detected. Specific tumour markers in the blood can also give relevant hints for cancer. An MRI of the head should be performed for patients with advanced-stage carcinoma or those with signs or symptoms of brain metastasis.

The findings for the characterization of the lung tumours are based on the evaluation of the autopsy findings as well as of the resections or materials gained during the treatment. Pathologists examine materials such as sputum, lavage, effusion fluid, and samplings of the tumour through bronchoscopy, thoracoscopy, mediastinoscopy, transbronchial biopsy, per thoracic fine needle biopsy for the cyto-pathological examinations to ensure the diagnosis, to determine whether the tumour has spread to the lymph vessels. The diagnosis can be ensured by pathomorphological examination of bronchial or transbronchial biopsy samples, to distinguish cancer from inflammation

or degenerative origin.[18]

There are many important aspects of the diagnosis, where the following clinical examinations must be carefully considered:

- Biochemical investigations, such as laboratory findings, and tumour markers.
- Imaging tests such as:
  - Chest X-ray
  - High-resolution Computer tomography (HRCT)
  - Sonography to exclude intraabdominal metastasis
  - CT angiography (CTA) to evaluate resectability
- Nuclear medical investigations such as:
  - Bone scan
  - Positron emission tomography CT (PET CT) to exclude extrapulmonary metastasis
- Lung function with perfusion scintigraphy to evaluate resectability

The topography of lung cancer is important for pathogenesis concerning early detection, operation, and metastatic pattern.[46][18]

2.5 Histology and Classification

Lung adenocarcinoma is diagnosed by cytology or small biopsy samples, as well as by surgical specimens during the operation. It manifests in various patterns and degrees of differentiation including lepidic, acinar, papillary, micropapillary, and solid with or without mucin formation. It also primarily occurs in mixed morphology in relation to the five most important histological patterns mentioned above.[47] The most frequently diagnosed predominant pattern is acinar, the second is solid or papillar, followed by lepidic and micropapillary.[47]

The invasive pulmonary adenocarcinomas were reclassified in 2015 by the International Association for the Study of Lung Cancer, American Thoracic Society, and European Respiratory Society according to their predominant architecture into lepidic, acinar, papillary, micropapillary, cribriform, and solid and in all of them mucin production can be found.[48][10] The term bronchioloalveolar carcinoma (BAC) has been replaced by lepidic pattern according to the International Association for the Study of Lung Cancer (IASLC), American Thoracic Society (ATS) and European Respiratory Society (ERS) in 2011. These are carcinomas with mucinous and nonmucinous subtypes, which show pneumonia-like infiltration more often than the nodular formation of bronchogenic carcinoma and are defined as a noninvasive adenocarcinoma without stromal, vascular, or pleural invasion.[49] In the new WHO classification invasive mucinous adenocarcinoma has abundant mucin and has a columnar or goblet cell morphology, which shows lepidic, acinar, papillary, micropapillary, cribriform, or solid patterns. This could give prognostic information. In the new classification, solid LUAD have either "mucin production" or they show adenocarcinoma markers which is in contrast to the previous edition. Invasive mucinous together with enteric and colloid LUAD are variants of lung adenocarcinoma.[50] Signet ring cell adenocarcinoma is no longer a subtype of LUAD but rather they are considered as a variant with signet ring features. In the new classification, mucinous cystadenocarcinoma has been merged with colloid LUAC.[10] Lung tumours, especially lung adenocarcinoma are classified in 2021 by the WHO[50] into: (The carcinomas are highlighted in bold.)

- Epithelial tumours
  - Papillomas
    - Bronchial papillomas
  - Adenomas
    - Sclerosing pneumocytoma
    - Alveolar adenoma
    - Papillary adenoma of the lung
    - Bronchiolar adenoma / ciliated muconodular papillary tumour
    - Mucinous cystadenoma of the lung

    - Mucous gland adenoma of the lung
- Precursor glandular lesions
    - Atypical adenomatous hyperplasia of the lung
    - Adenocarcinoma in situ of the lung
- **Adenocarcinomas**
    - **Minimally invasive adenocarcinoma of the lung**
        - **Minimally invasive adenocarcinoma, non-mucinous**
        - **Minimally invasive adenocarcinoma, mucinous**
    - **Invasive non-mucinous adenocarcinoma of the lung**
        - **Lepidic adenocarcinoma**
        - **Acinar adenocarcinoma**
        - **Papillary adenocarcinoma**
        - **Micropapillary adenocarcinoma**
        - **Solid adenocarcinoma**
    - **Invasive mucinous adenocarcinoma of the lung**
        - **Invasive mucinous adenocarcinoma**
        - **Mixed invasive mucinous and non-mucinous adenocarcinoma**
    - **Colloid adenocarcinoma of the lung**
    - **Fetal adenocarcinoma of the lung**
    - **Enteric-type adenocarcinoma of the lung**
- Squamous precursor lesions
    - Squamous dysplasia and carcinoma in situ of the lung
- **Squamous cell carcinomas**
    - **Squamous cell carcinoma of the lung**
    - **Lymphoepithelial carcinoma of the lung**

  - **Large cell carcinomas**
    - **Large cell carcinoma of the lung**
  - **Adenosquamous carcinoma**
    - **Adenosquamous carcinoma of the lung**
  - **Sarcomatoid carcinomas**
    - **Pleomorphic carcinoma of the lung**
    - **Pulmonary blastoma**
    - **Carcinosarcoma of the lung**
  - **Other epithelial tumours**
    - **NUT carcinoma of the lung (see NUT carcinoma of the thorax)**
    - **Thoracic SMARCA4-deficient undifferentiated tumour**
  - Salivary gland-type tumours
    - Pleomorphic adenoma of the lung
    - **Adenoid cystic carcinoma of the lung**
    - **Epithelial-myoepithelial carcinoma of the lung**
    - **Mucoepidermoid carcinoma of the lung**
    - **Hyalinizing clear cell carcinoma of the lung**
    - **Myoepithelioma and myoepithelial carcinoma of the lung**

- Lung neuroendocrine neoplasms
  - Precursor lesion
    - Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia
  - Neuroendocrine tumours
    - Carcinoid/neuroendocrine tumour of the lung
  - **Neuroendocrine carcinomas**
    - **Small cell lung carcinoma**
    - **Large cell neuroendocrine carcinoma of the lung**

- Tumours of ectopic tissues
  - Melanoma of the lung
  - Meningioma of the lung
- Mesenchymal tumours
  - Pulmonary hamartoma
  - Pulmonary chondroma
  - Diffuse pulmonary lymphangiomatosis
  - Pleuropulmonary blastoma
  - Pulmonary artery intimal sarcoma
  - Congenital peribronchial myofibroblastic tumour
  - Primary pulmonary myxoid sarcoma with EWSR1-CREB1 fusion
  - PEComatous tumours:
    - Lymphangioleiomyomatosis of the lung
    - PEComa of the lung
- Haematolymphoid tumours
  - MALT lymphoma of the lung
  - Pulmonary diffuse large B-cell lymphoma
  - Lymphomatoid granulomatosis of the lung
  - Intravascular large B-cell lymphoma of the lung
  - Pulmonary Langerhans cell histiocytosis
  - Pulmonary Erdheim-Chester disease

It is also important for the therapy to distinguish between small cell and non-small cell tumours.[18] Small cell lung cancers are poorly differentiated neuroendocrine tumours. They are aggressive and often diagnosed very late. Long time survival is approximately 10-15%. Non-small cell lung cancer are squamous cell carcinomas (LSCC), adenocarcinomas and large cell carcinomas. They have a moderate growth tendency. After resection, they have long overall survival. Immune chemotherapy is the standard

therapy for unresectable tumours. There are also mixed forms of carcinoma with small and large cell components. The therapy is according to the predominant component.

Lung adenocarcinoma is a non-small cell lung carcinoma with histologically larger cells. It has typical histological cell changes with glands-like (secretory) qualities, as well as certain architectural, cytological, and molecular features or in the case of mucinous lung adenocarcinomas, which produce lots of mucus. If there is a low grade of differentiation, the tumour cells are similar to normal lung tissue.

One-third of lung adenocarcinoma cases differentiate along a mucinous pathway as atypical goblet cell proliferation (Figure 2.1).[19][30][10]

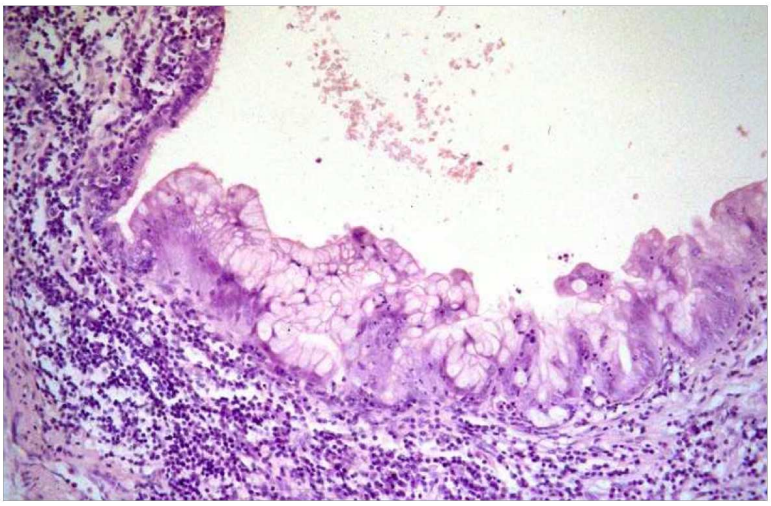

Figure 2.1: Atypical goblet cell proliferation[10](Reproduced with permission from Springer Nature)

Mucinous AC originates most probably from basal/peripheral stem cells, which can produce different types of mucins, such as fucosylated and sialylated carbohydrates, acidic sulfated carbohydrates, and glycosylated lipoproteins.[29][10]

In our study we distinguish the mucinous adenocarcinoma from non-mucinous adenocarcinomas not only by mucin production in H&E but also through positivity for mucicarmine. Mucicarmine is a product binding mucopolysaccharides and visualizing them, as well as through Alcian blue staining of at least 80% of tumour cells, which is much more sensitive than through only abundant mucin production. Mucin production

does not depend on subtypes such as acinar, papillar, etc.

In the histology images of non-mucinous and mucinous lung adenocarcinoma, you can observe the differentiation of lepidic, acinar, papillary, micropapillary, and solid types without mucin production in non-mucinous adenocarcinoma. Conversely, abundant mucin production in the histology images of mucinous lung adenocarcinoma is evident.[51]

The TNM-Classification according to UICC describes the tumour stages of malignant diseases for further therapy options. It uses clinical and histological investigations for ordering and classification of the tumour. The tumours are classified into stages Ia-IV according to size, topography, and extent as well as according to lymph node and distant metastasis. This classification is very crucial for therapy and prognosis.[52][18] Low-stage tumours are confined in the lung and are small, high-stage tumours are large and spread metastasis in the periphery.

Mucinous differentiation is already present in the precursor lesions as an atypical goblet cell proliferation (Figure 2.1), although the precursor lesion for mucinous adenocarcinoma is not clearly defined. The origin is probably basal or peripheral stem cells capable of producing different types of mucins, such as fucosylated and sialylated carbohydrates, acidic sulfated carbohydrates, and glycosylated lipoproteins. It seems that a certain inflammatory phenotype as a response to tobacco smoking leads to this specific mucinous precursor lesion, which later undergoes adenocarcinoma development. As the non-mucinous variants also mucinous adenocarcinomas are predominantly peripheral carcinomas. When looking up mucinous adenocarcinomas in a daily practice, it became evident, that there are also architectural patterns as well as cell types, which might have their own prognostic value.[53] In our study, we also wanted to clarify these questions.

2.6 Histochemistry and Immunohistochemistry

Histochemistry (HC) and immunohistochemistry (IHC) are essential tools in the diagnosis and classification of carcinomas. IHC involves the binding of antibodies to specific

antigens, which can typically be detected or examined using a light microscope. IHC is used not only for the classification of tumours but also for determining their origin, for example, in identifying metastatic tumours. In some cases, it is very difficult to distinguish the primary lung adenocarcinoma from lung metastasis, for instance when it comes to adenocarcinomas of the gastrointestinal tract, a corpus carcinoma of the uterus, or a kidney carcinoma. The origin of the tumour can be found with immunohistochemical investigations on paraffin slides. In many cases, it is possible to determine metastasis from the thyroid, prostate or liver, and other organs. If lung adenocarcinoma has a central scar, it is also difficult to distinguish it from a scarring carcinoma or poorly differentiated squamous cell carcinoma. Furthermore, IHC can be used to determine the expression of targetable proteins (e.g., EGFR in lung cancer, HER2 in breast cancer) or immune checkpoint proteins (e.g., PD-L1) to select appropriate targeted therapies or assess the suitability of immune checkpoint inhibitors. IHC can also help elucidate the interaction between the tumour and the immune system, which can lead to the development of immunotherapy strategies.[54][55][47][18]

Various biochemical substances can be detected in lung tumours by histochemical methods. For example, P63 is positive in all bronchial carcinoma, but also in 1/3 of lung adenocarcinoma. Adenocarcinoma of the lung is positive for thyroid transcription factor-1 (*TTF1*) and cytokeratin 7 (*CK7*) marker and negative for squamous cell carcinoma markers such as *CK5/6* and *p63*. A positive *TTF1* is proof of a primary lung adenocarcinoma. But it could also be a metastasis of a thyroid carcinoma. *TTF1* is positive in 80-85% of lung adenocarcinoma and it is also important for the prognosis, because tumours, which are negative for *TTF1*, have a worse prognosis.[56] There are also other important markers for lung adenocarcinoma such as *Ki67*, which is important in proliferation.

In our study we wanted to study the expression of the following substances in mucinous lung adenocarcinomas in correlation with prognosis and survival: *CK7* and *20*, *TTF1* and *CDX2* (colon cancer marker), and *MUC1*, *MUC2* and *MUC-5AC* proteins as well as *p14*, *p15*, and *p16* proteins.

2.7 Genetics

Genetic aberrations are one of the causes of the development of lung cancer. They also create possibilities for creating specific mutation-targeted therapies, which are already established as a new therapy form. Several genetic alterations were found, which induce goblet cell proliferations such as *MUC2* and *5AC* genes and also several cytokines such as *IL4*, *IL4R*, and *IL9*, *13* and *17*.[57][58][59][60][61][29][62] Tyrosine kinase domain mutations of the epidermal growth factor receptor (EGFR) gene occur frequently in lung adenocarcinoma patients. These patients respond often to tyrosine kinase inhibitor drugs such as gefitinib, or erlotinib. Unfortunately many of them especially smokers quickly develop resistance.[63]

KRAS mutations are more common in contrast to the *EGFR* mutations. First 1982 *RAS* mutations were described as a mechanism of oncogene activation following point mutations of certain codons within the family of *RAS* genes. *KRAS* mutation is detected in 30% of lung adenocarcinoma.[27] However, it is more commonly observed in lung adenocarcinoma patients with smoking history rather than in non-smokers. The patients are resistant to *EGFR*-tyrosin-kinase inhibitors.[63]

2007 Soda et al. described a translocation within chromosome 2p between the receptor tyrosine kinase gene *ALK* (anaplastic lymphoma kinase) and echinoderm microtubule-associated protein-like 4 (*EML4*) gene, building *EML4-ALK* fusion protein in 6,7% of non-small-cell-lung-cancer (NSCLC)[64] and estimated 3-6% in lung adenocarcinomas.[65]

Chromosomal rearrangements of the *ROS1* receptor tyrosine kinase gene are an oncogenic driver in lung cancer, presenting in 1-2% of patients. The results of therapeutic interventions sound promising.[66]

In our study, we looked for possible rearrangements in *EML4ALK* and *ROS1* as well as *KRAS* mutational status in mucinous lung adenocarcinoma and correlated these with survival and prognosis.

2.8 Therapy

The treatment of lung cancer is decided individually according to tumour stage, histology, patient condition, age, pulmonary function, and co-morbidities. Malignancies should be treated multi-modal with surgery, chemotherapy, radiotherapy, and other specific therapy options.

The TNM classification of the tumour is crucial for the decision of the therapy. If the patient received operative measures the surgical specimen have also to be examined by the pathologist to update and plan further therapeutic procedures.[18]

The surgical treatment of patients is the primary treatment option in cases of operable tumours, however, only 25-30% of the patients are in operable condition at the time of diagnosis. An increasing number of patients are still treated with curative intention.[67][47] The others could not be operated on for functional reasons or because of distant metastases or local tumour extension, so about 75% are in a high-stage non-resectable. If the tumour is only located in the lung, the tumour should be operated through a thoracotomy or median thoracotomy or through video-assisted thoracic surgery (VATS). If the tumour is invasive and spreading in the surrounding tissues neoadjuvant chemotherapy or radiotherapy should be done. Chemotherapy and radiotherapy slow the progression of the tumour and reduce the pain. Chemotherapy can be used before or after surgical therapy or combined with other therapy options like chemoradiotherapy.

The resectability criteria of lung cancer are:

- Functional criteria
  - Surgical intervention must be cardiopulmonary tolerable
- Oncological criteria
  - No distant metastases
  - No node involvement (extrathoracic, contralateral, paratracheal, ipsilateral) - exception: bronchial cancer with only primary cerebral metastasectomy.

- Local criteria
  - No tumour invasion of mediastinal structures, in the large vessels of the pleural dome
  - Tumour infiltration in the bronchial system cranially must not be more than 2 cm distal to the main bifurcation

The preoperative and postoperative treatment with chemotherapy and/or radiation therapy (multimodal) to support the surgical approaches is also very important for a good prognosis. The postoperative course depends on lymph node involvement, the presence of microvascular invasion, and tumour stage. The most used chemo drugs are cisplatin, carboplatin, paclitaxel, albumin-bound paclitaxel, docetaxel, gemcitabine, vinorelbine, irinotecan, etoposide, vinblastine, pemetrexed. Chemotherapy is well known to cause a plethora of adverse events such as hair loss, aphthae, loss of appetite, nausea, vomiting, diarrhea or constipation, increased chance of infections, easy bruising or bleeding, and fatigue. Some side effects are specific to some drugs for example cisplatin can cause nerve damage, called peripheral neuropathy.

There are 2 main radiation therapy forms for lung adenocarcinoma - external beam radiation therapy from outside and internal radiation therapy called brachytherapy, which is applied through bronchoscopy or during the operation. Radiation therapy uses high-energy rays such as X-rays or particles to kill cancer cells. It is often used if the tumour is not possible to remove surgically. There are lots of side effects such as fatigue, nausea and vomiting, loss of appetite and weight loss, skin changes and hair loss in the area being treated, esophageal damage with dysphagia, cough, and breathing problems.

There are also other new specific therapy options like immunotherapy, which has become part of the established therapy for lung adenocarcinoma. The immunotherapeutics used include Pembrolizumab (Keytruda), Nivolumab (Opdivo), Atezolizumab (Tecentriq), and Ipilimumab (Yervoy). These therapies can be used as various lines of treatment, either in combination or as monotherapies. These immunotherapeutics target key immune checkpoints (PD-1, PD-L1, and CTLA-4), enhancing the immune response against lung adenocarcinoma cells and improving patient outcomes.[68][69][70]

Lung tumour ⇒ Malignancy? ⇒ Biopsy/Sampling ⇒ Pathology ⇒ Diagnosis ⇒ Staging ⇒ Therapy ⇒ Further procedure according to development of the disease

Above we see the rough algorithm for the treatment of lung cancer.

The new concepts in the research and treatment of lung adenocarcinoma contribute an important change to the clinical practice for improving patient care and outcomes. For example the revised growth patterns with histological subtyping and the integrated diagnosis, where histological features with molecular and genetic data provide a comprehensive understanding of the tumour and guide treatment strategies. There are also other advances concerning targeted therapies for specific genetic mutations in lung adenocarcinomas like EGFR, ALK, ROS1, and BRAF. Or Immunotherapy with checkpoint inhibitors with biomarkers like PD-L1 expression. Or Comprehensive Genomic Profiling with next-generation sequencing (NGS) which allows detailed genetic profiling, identifies actionable mutations, and enables personalized treatment.[71][72][73][74]

There are several screening programs concerning lung adenocarcinoma to improve early detection, reduce mortality, and improve patient outcomes and survival. These programs focus on identifying individuals at high risk for developing lung cancer based on factors such as age, smoking history, and other risk factors. Screening tests, such as low-dose computed tomography (CT), are used to detect early-stage lung cancer. Regular screenings at specified intervals, following established guidelines and recommendations, are important. It is also important to provide information about the risks and benefits of screening, as well as counseling concerning smoking cessation.[75][76][77]

2.9 Prognosis

Malignant lung tumours have a poor prognosis. The life expectation of lung cancer patients depends on lymph node involvement, the presence of microvascular invasion and tumour stage as well as from patient's overall health situation. The average 5 years survival rate of primary lung cancer is 10-15%[78][79] and the 10-year survival rate is

8-10%[80][81], however 5 years survival rate can increase up to 35-40% if the tumour can be resected in an early stage, survival rate is slightly better with 5 years survival of 85% in patients under the age of 30.[82]
Up to 75% of the resected patients die within the first 5 postoperative years.[82] The resected peripheral tumours in an asymptomatic stage have a good prognosis, and so adenocarcinoma in situ (AIS) and minimally invasive adenocarcinomas have 100% 5-year disease-free survival. The disease-free survival for stage I lung adenocarcinoma is between 67-90%, depending on the histological subtype.[83]

Lung adenocarcinoma patients can only be cured if the entire tumour is surgically removed or destroyed with radiotherapy, but mostly the tumour is diagnosed at a stage when this is not possible. Less than one-fifth of the patients survive five years. The prognosis of the mucinous adenocarcinoma of the lung is poorly published. According to a study from 2011 with 210 patients five-year survival rate for minimally invasive adenocarcinoma and lepidic-predominant adenocarcinoma are approaching 100%, whereas micropapillary-predominant and solid with mucin-predominant adenocarcinoma are associated with poor prognosis, with 5-year survival rate of 38%. Papillary- and acinar-predominant lung adenocarcinomas have an intermediate prognosis of 50 to 70%.[20][47]
According to screening studies stopping smoking reduces mortality.[84]

3 Study

It is a generally accepted assumption, that mucinous adenocarcinomas if stratified by stage behave more aggressively than their non-mucinous counterparts, cytomorphologic and architectural features have not been evaluated and correlated with prognosis.[33][34][35] Therefore we aim to address these features in over 70 cases derived from one institute.

The aim of the study is to compare the survival rate as well as the progression-free time of mucinous versus non-mucinous adenocarcinomas of the lung and to detect the influence of various factors for example smoking behavior, therapy ...etc. on the survival rate of the mucinous and non mucinous adenocarcinoma of the lung. Even several co-morbidities have been observed. It is an evaluation of the survival of patients with adenocarcinoma of the lung and the influence of smoking and pack-years, chemotherapy, radiotherapy, metastasis, survival, co-morbidities, and postoperative complications. Smoking has a bad influence on the development of adenocarcinoma of the lung, chemotherapy and radiotherapy should prolong the survival rate of the patient. This study aims also to investigate co-morbidities and their association with tumour development and prognosis. Lower TNM (Tumour Nodes Metastasis) stage at the time of initial diagnosis will lead to a better outcome. Hence what was expected from the study is to find out the correlation of the above-mentioned factors in tumour development and progression. The second aim of the study is the investigation of the various causes that lead to death or can have an impact on the extension or shortening of the lifetime of the patient.

Further aims are the production of a clinical database for translational research in lung adenocarcinomas.

The survival rate and time to progression in patients with mucinous versus non-mucinous adenocarcinoma of the lung should be determined.

3.1 Materials and Methods

3.1.1 Clinical data

The clinical data of 76 patients with mucinous and 259 patients with non-mucinous adenocarcinoma of the lung (a total of 335 patients), were collected retrospectively from the Diagnostic and Research Institute of Pathology as well as from the Division of Thoracic and Hyperbaric Surgery, Medical University of Graz. Staging was available for all cases. All these patients underwent surgical treatment for their disease. The data included age, gender, smoking status (pack years), date of surgery, postoperative course, recurrence during follow-up visits, disease (overall) free or progression-free survival, metastasis (including location), death of disease, other non-tumour related diseases (comorbidities, etc). We collected the clinical data of the patients from MEDOCS, the medical data system of patients of Styria, and patient files.

Among the 335 cases of surgically resected pulmonary adenocarcinomas, a subset of 76 cases were classified as mucinous adenocarcinomas and obtained from our lung biobank of the Diagnostic and Research Institute of Pathology, Medical University of Graz. Mucinous adenocarcinoma diagnosis was confirmed through positive mucicarmine and Alcian blue staining in at least 80% of tumour cells, indicating a more precise evaluation compared to the mere presence of visible mucin production. Additionally, the diagnosis was supported by either columnar or goblet cell morphology, or the presence of characteristics associated with the enteric or colloid adenocarcinoma. These patients underwent surgical removal of the adenocarcinomas at a stage that allowed for operability, both clinically and pathologically.

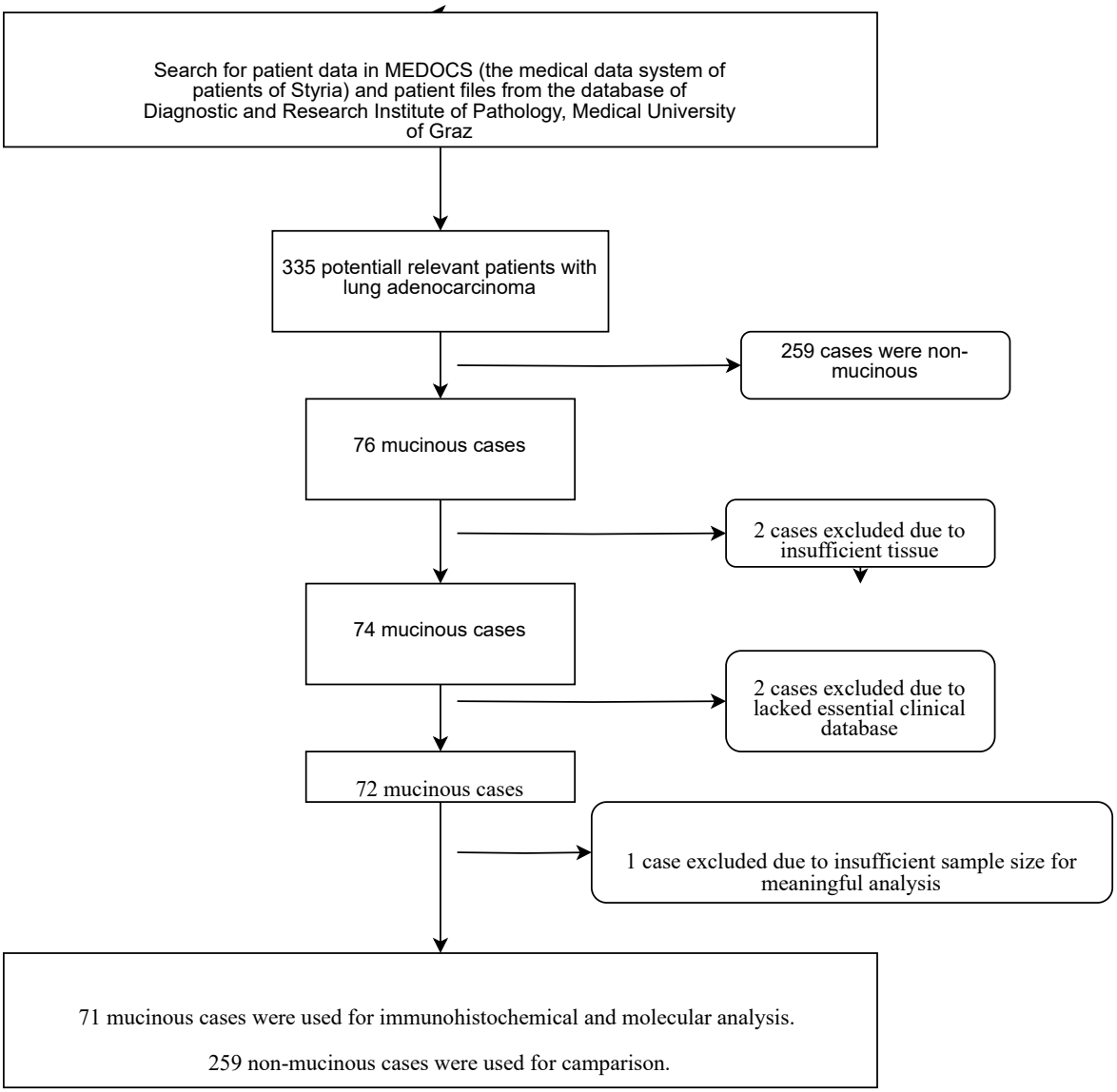

Figure 3.1: Flow chart with excluded/included cases.

We obtained the tissue blocks from the Lung Biobank for analysis. Two cases were excluded from the study due to insufficient tissue, while another two cases lacked essential clinical data and were also excluded. Additionally, a single case of surgically resected enteric adenocarcinoma (AC) was identified but excluded from the analysis due to an insufficient sample size for meaningful analysis. Consequently, a total of 71 cases of mucinous AC were included in the immunohistochemical and molecular analysis (Figure 3.1). Thin sections, measuring four micrometers in thickness, were prepared from formalin-fixed paraffin-embedded tissue blocks. These sections were subsequently

stained with hematoxylin and eosin (H &E), as well as mucicarmine and Alcian blue at pH 2.5 for further evaluation. Detailed patient information can be found in Table 3.10, with additional Tables 3.12 and 4.1 providing more comprehensive details.[10]

Patients were grouped into clinically homogenous groups according to their TN stage: Group 1 = T1 or T2 and N0 (T1/T2+N0); Groups 2 = T1 or T2 and N1 or N2 (T1/T2+N1/N2); Groups 3 = T3 or T4 and any N-group (T3/T4+N?), according to the UICC/TNM staging system into Stage IA-IV (Tables 3.10 and 3.12).

3.1.2 Patterns of mucinous adenocarcinomas

Initially, all mucinous cases underwent a reevaluation conducted by two independent pathologists (UGM, HHP). The groups were homogenized and the lung adenocarcinomas (LUACs) were staged using the 7th edition of the TNM Staging System.[52] The classification process involved the application of the same architectural patterns utilized for non-mucinous adenocarcinomas, including acinar, papillary, micropapillary, solid, cribriform, and colloid patterns. Additionally, mucinous cystadenocarcinomas and adenocarcinomas with signet ring cell morphology were also considered. The presence of diffuse spreading without the formation of a pseudo capsule defined colloid adenocarcinomas, while cystadenocarcinomas exhibited a distinct pseudo capsule. A mixed pattern resembling the recommendations for non-mucinous adenocarcinomas was also observed. The assessment of surface percentage involved the identification of secondary and tertiary patterns, recorded in increments of 20% for primary and secondary patterns. We preferred using 20% increments due to their practicality, as this approach is easier and more manageable for pathologists during routine diagnostic work. It is also sufficient to capture clinically relevant differences between tumor types or patterns and ensures greater consistency and reproducibility among different pathologists. Overall, this method speeds up the evaluation process while still providing adequate information for clinical decision-making and the study. Any secondary component observed was also documented. Furthermore, various types of mucin storage were recorded, such as the goblet cell type characterized by intracytoplasmic mucin droplets and extracellular mucin production seen in colloid adenocarcinomas.

Colloid LUAC is defined as containing tumour cell groups isolated in extracellular mucin, which can be micropapillary or papillary and they have a second pattern, most frequently acinar and because of their mucin production, they fulfill the criteria of our cohort. They can grow nodular or diffuse like invasive mucinous LUAC.[85]

3.1.3 Mucin storage and release

There were four distinct types of mucin storage and secretion observed. The goblet cell type exhibited large supranuclear vacuoles that secreted mucin apically into acinar lumina, representing luminal/apical secretion. In the columnar cell type, very small vacuoles were responsible for storing mucin, which was then secreted apically into acinar lumina. In colloid adenocarcinomas and cystadenocarcinomas, a third type of mucin storage was identified. In these cases, tumour cells stored mucin in small cytoplasmic vacuoles without their basoapical orientation. The secretion of mucin occurred basolaterally into the stroma, resulting in the presence of abundant mucin within the tumour (basolateral secretion). In signet ring cell adenocarcinomas, a fourth type of mucin storage and secretion was observed. In this case, intracytoplasmic lumina formed within individual tumour cells, serving as sites for mucin secretion (intracytoplasmic luminal secretion).[10]

Furthermore, we assessed the type of mucin storage and release, including the goblet cell type, intracellular storage, and extracellular release.

3.1.4 Immunohistochemistry - MUC proteins

We utilized 71 tissue blocks to construct a tissue microarray, which served as the basis for the molecular analysis. The tissue microarray consisted of a recipient paraffin block containing at least three cores of tumour tissue and one core of uninvolved normal lung tissue, which were carefully separated from the tumour area. Immunohistochemistry was performed on 4 μm sections using specific antibodies, including *CK7* and *20*, *TTF1*, *CDX2*, *MUC1*, *MUC2*, *MUC5AC*, *p14ARF*, *p15INK4B*, *p16INK4A*, *ALK1*, and *ROS1* (Table 3.11). The immunohistochemical stains were evaluated semi-

quantitatively using an intensity score ranging from 0 to 3+, which was then multiplied by the percentage of positively stained tumour cells in 10% increments to obtain an H-score ranging from 0 to 300. The mean H-score was calculated based on the evaluation of the three cores.

To evaluate the reliability of tissue microarray (TMA) sampling, full sections from 20 randomly selected cases were stained for *MUC1*, *TTF1*, *p14*, *p16*, and *ALK1*.[10]

3.1.5 KRAS Mutation

In the analysis of *KRAS* mutations, whole tumour tissue sections from 71 pre-treatment adenocarcinomas were utilized. Sections measuring four to six micrometers in thickness were dewaxed in xylene. Tumour areas marked on the slides were carefully scraped. Genomic DNA was extracted from the paraffin-embedded tissues using the EZ1 DNA investigator Kit from Qiagen (Hilden, Germany). Subsequently, *KRAS* mutation analysis was performed using the Therascreen® *KRAS* PYRO® Kit on the PyroMark Q24 instrument, following the manufacturer's instructions.

3.1.6 EML4ALK and ROS1 Rearrangement

Initially, tissue sections were stained using an ALK1 antibody (listed in Table 3.11), resulting in positive staining in three cases. Subsequently, fluorescence in situ hybridization (FISH) analysis was conducted using the FISH break-apart probe from Vysis (Abbott Laboratories, Abbott Park, IL, USA) to further evaluate these cases. Regarding *ROS1* translocations, an antibody specific to *ROS1* (listed in Table 3.11) was utilized. However, all the cases showed negative results, leading to the decision not to proceed with further evaluation by FISH.[10]

The research conducted in this study received approval from the Ethical Committee of the Medical University (Approval No. 24-135 ex 11/12).

3.2 Statistical Analysis

The clinical data was evaluated with the R software (www.r-project.org). The obtained results indicate graphical and tabular as well as standard errors. The values lower 95%CI and upper 95% CI P-value below 0.05 were regarded as statistically significant.[10]

The Kaplan-Meier method was used to calculate the survival rates. Then the long-rank test was used to compare the survival rates between the groups. These survival rates were adjusted by Cox regression in order to include the risk factors. After that modification, these were then tested by Wald test criterion

The paired t-test was employed to assess the expression levels of various AC markers (*CK7*, *CK20*, *TTF1*, *CDX2*, *MUC1*, *MUC2*, *MUC5AC*, *p14*, *p16*) across different variants of mucinous adenocarcinomas.[10]

3.3 Results

In the following diagram, we can see the survival analysis of 335 adenocarcinoma patients in accord with T Stage (T Stage 1, T Stage 2, T Stage 3, and T Stage 4), how long the patients lived or have been observed when they are still alive, whether they died at the end, and which T stage they have. These are the first results graphically, the colours represent the individual T stages. There was a statistically significant difference between T-stages (log rank test; P= 0.00067).

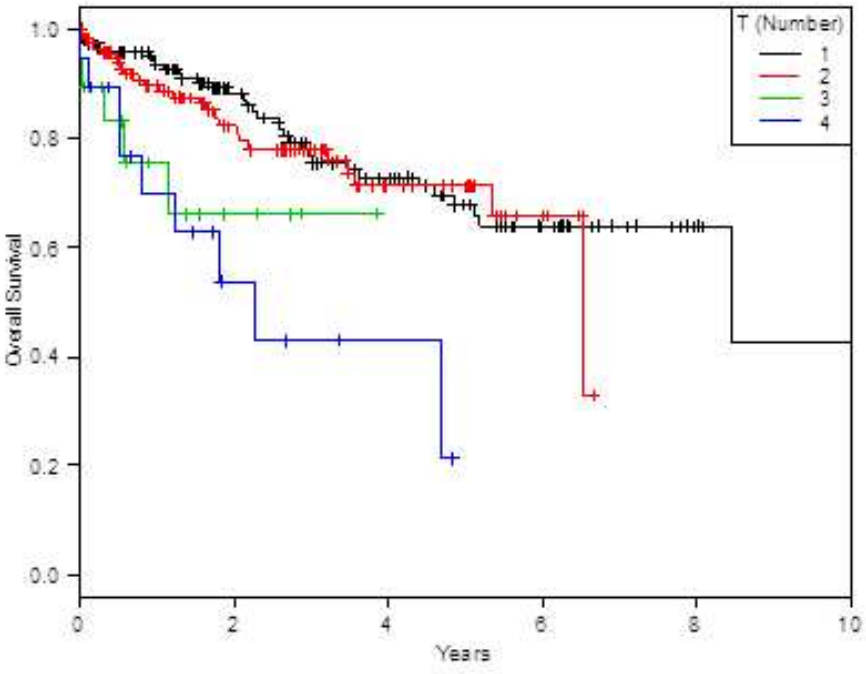

Figure 3.2: The various coloured curves represent distinct survival analyses based on the stages of tumour size.

There was a statistically significant difference between T- (log-rank test; P= 0.00067) and N-stages (log-rank test; P= 0.000073). The corresponding hazard ratio of mucinous carcinoma was 1.31 (95%CI: 0.77 to 2.20). The relative risk of 1 would be neutral. There was no difference between mucinous and non-mucous carcinoma with respect to survival after adjusting for clinical groups, however, the confidence interval was now 0.63 to 1.83.

Mucinous differentiation in adenocarcinomas does not influence survival. They behave biologically similar. The LN-metastasis is equally common in both types and even if the patients are grouped with the LN-metastasis in both groups the outcome is the same. There was a statistically significant difference between T- (log-rank test; P= 0.00067) and N-stages (log-rank test; P= 0.000073). The corresponding hazard ratio of mucinous carcinoma was 1.31 (95%CI: 0.77 to 2.20). The relative risk of 1 would be neutral. There was no difference between mucinous and non-mucous carcinoma with respect to survival after adjusting for clinical groups, however, the confidence interval was now 0.63 to 1.83.

In the following table of the diagram, you can read the table value at a certain time.

T.char1=1

time	n.risk	n.event	survival	std.err	lower 95% CI	upper 95% CI
0	148	0	1.000	0.0000	1.000	1.000
2	82	15	0.881	0.0292	0.826	0.940
5	37	16	0.679	0.0508	0.586	0.786

T.char1=2

time	n.risk	n.event	survival	std.err	lower 95% CI	upper 95% CI
0	118	0	1.000	0.000	1.000	1.000
2	57	17	0.822	0.040	0.748	0.905
5	19	6	0.713	0.055	0.613	0.830

T.char1=3

time	n.risk	n.event	survival	std.err	lower 95% CI	upper 95% CI
0	18	1	0.947	0.0512	0.852	1.000
2	4	4	0.661	0.1299	0.450	0.971

T.char1=4

time	n.risk	n.event	survival	std.err	lower 95% CI	upper 95% CI
0	18	1	0.947	0.0512	0.852	1.000
2	5	6	0.538	0.1340	0.330	0.877

Table 3.1: The four tables titled "T.char1 1-4" provide information on tumour stages 1-4. Each of them has the following columns. The first column named "time" represents time in years. The second column named "n-risk" displays the number of patients within that specific time period. The third column named "n.event" indicates the number of patients who discontinued their participation in the study due to various reasons, such as death. The fourth column named "survival" shows the survival rate as a percentage. The fifth column named "std. err" presents the standard deviation. The sixth column named "lower 95% CI" displays the confidence interval below 95%. The seventh column named "upper 95% CI" displays the confidence interval above 95%.

The following line is the result of a statistical test. (Score (log-rank) test = 17.08 on 3 df, p=0.00067) This test is positive and the 0-hypothesis is rejected, there is a

difference between the curves because the P-value is below 0.05.

In the N stages, there are two things we have done. First, we only examined N and divided patients into groups according to their TN stage: Group 1 = T1 or T2 and N0 (T1/T2 and N0); Group 2 = T1 or T2 and N1 or N2 (T1/T2 and N1/N2); Group 3 = T3 or T4 and any N-group (T3/T4 and N?).

There was a statistically significant difference between N-stages (log-rank test; P= 0.000073).

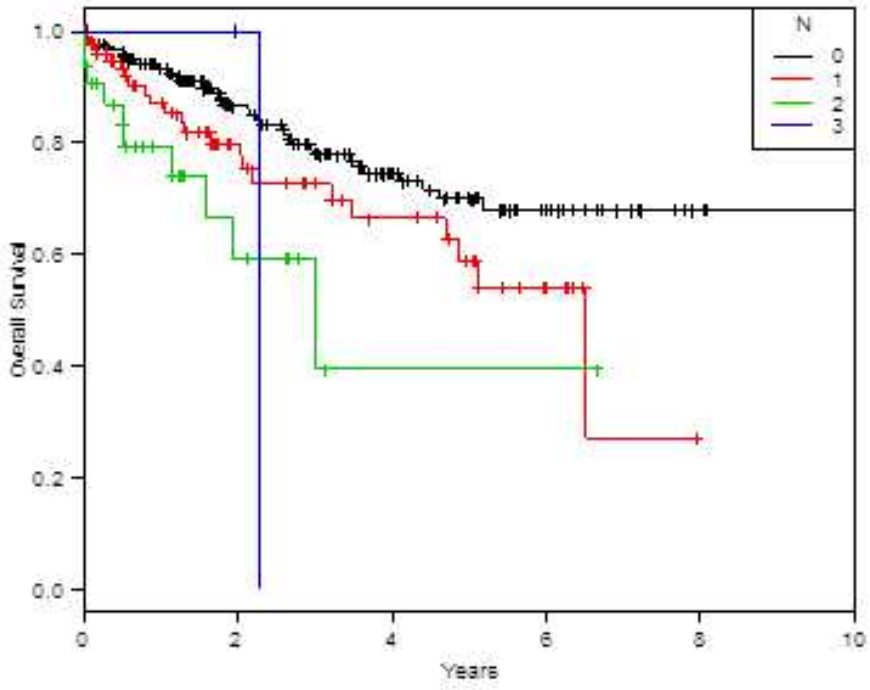

Figure 3.3: The various coloured curves represent distinct survival analyses based on the stages of lymph node involvement.

37 observations deleted due to missingness

factor(N.Stadium)=0

time	n.risk	n.event	survival	std.err	lower 95% CI	upper 95% CI
0	183	0	1.000	0.0000	1.000	1.000
2	105	21	0.867	0.0274	0.815	0.922
5	39	16	0.700	0.0448	0.618	0.794

factor(N.Stadium)=1

time	n.risk	n.event	survival	std.err	lower 95% CI	upper 95% CI
0	79	0	1.000	0.0000	1.000	1.000
2	34	13	0.799	0.0506	0.706	0.905
5	14	7	0.590	0.0797	0.453	0.769

factor(N.Stadium)=2

time	n.risk	n.event	survival	std.err	lower 95% CI	upper 95% CI
0	30	2	0.938	0.0428	0.857	1.000
2	8	7	0.594	0.1165	0.404	0.872
5	1	1	0.396	0.1793	0.163	0.962

factor(N.Stadium)=3

time	n.risk	n.event	survival	std.err	lower 95% CI	upper 95% CI
0	2	0	1	0	1	1
2	1	0	1	0	1	1

Score (logrank) test = 19.07 on 2 df, p=0.000073

Table 3.2: The four tables titled "factor(N.Stadium) 0-3" provide information on lymph node stages 0-3. Each of them has the following columns. The first column named "time" represents time in years. The second column named "n-risk" displays the number of patients within that specific time period. The third column named "n.event" indicates the number of patients who discontinued their participation in the study due to various reasons, such as death. The fourth column named "survival" shows the survival rate as a percentage. The fifth column named "std. err" presents the standard deviation. The sixth column named "lower 95% CI" displays the confidence interval below 95%. The seventh column named "upper 95% CI" displays the confidence interval above 95%.

Above we see that in both, the non-mucinous adenocarcinomas and mucinous variants, the T and N stages are significantly associated with survival.

Survival in clinically homogenous groups according to their TN stage: A test was also done with clinically homogenous groups with the results shown in the table and the diagram below.

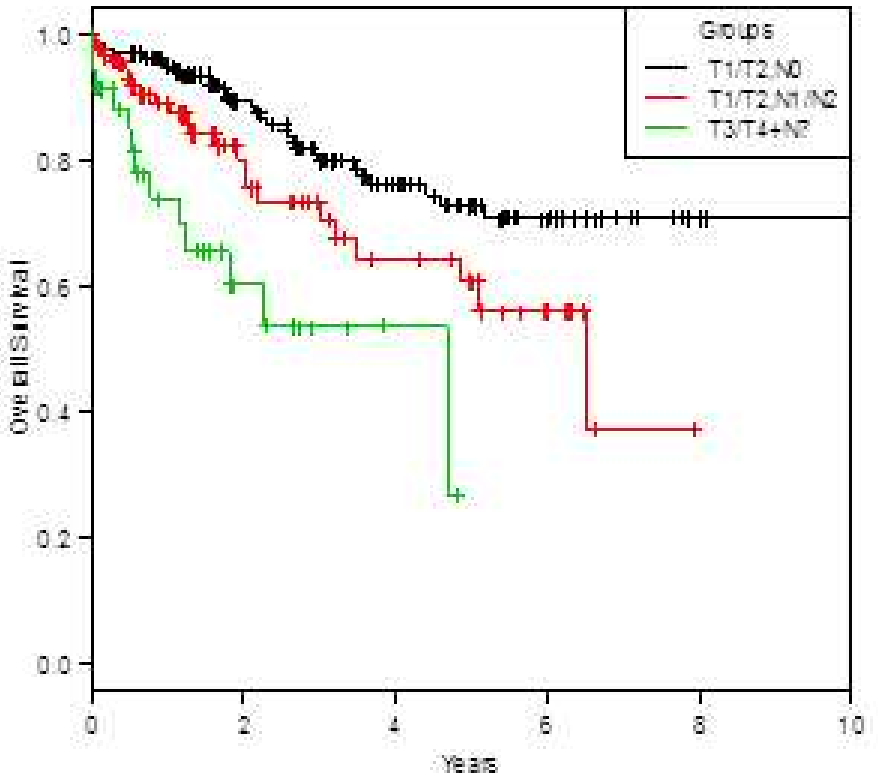

Figure 3.4: The various coloured curves represent distinct survival analyses based on groups according to the UICC TNM Classification of lung adenocarcinoma.

44 observations deleted due to missingness
Groups=T1/T2,N0

time	n.risk	n.event	survival	std.err	lower 95% CI	upper 95% CI	
0	162	0	1.000		0.0000	1.000	1.000
2	99	15	0.893		0.0264	0.843	0.946
5	39	15	0.729		0.0452	0.645	0.823

Groups=T1/T2,N1/N2

time	n.risk	n.event	survival	std.err	lower 95% CI	upper 95% CI	
0	92	0	1.000		0.0000	1.000	1.000
2	38	14	0.803		0.0487	0.713	0.905
5	15	7	0.614		0.0750	0.483	0.780

Groups=T3/T4+N?

time	n.risk	n.event	survival	std.err	lower 95% CI	upper 95% CI	
0	33	2	0.943		0.0392	0.869	1.000
2	9	9	0.603		0.0978	0.438	0.828

Table 3.3: The three tables above show groups according to the UICC TNM Classification of lung adenocarcinoma, respectively. Each of them has the following columns. The first column named "time" represents time in years. The second column named "n-risk" displays the number of patients within that specific time period. The third column named "n.event" indicates the number of patients who discontinued their participation in the study due to various reasons, such as death. The fourth column named "survival" shows the survival rate as a percentage. The fifth column named "std. err" presents the standard deviation. The sixth column named "lower 95% CI" displays the confidence interval below 95The seventh column named "upper 95% CI" displays the confidence interval above 95%.

In summary, there was a statistically significant difference between T-stages (log-rank test; P= 0.00067). There was a statistically significant difference between N-stages (log-rank test; P= 0.000073). Thus, we conclude that survival depends on the T stages and N stages.

Then we differentiate between mucinous and non-mucinous tumours, illustrated by the red and the green graphs in the chart below. There is no significant difference between mucinous and non-mucinous adenocarcinoma of the lung, as the chart shows at first glance. Mucinous has no association with survival.

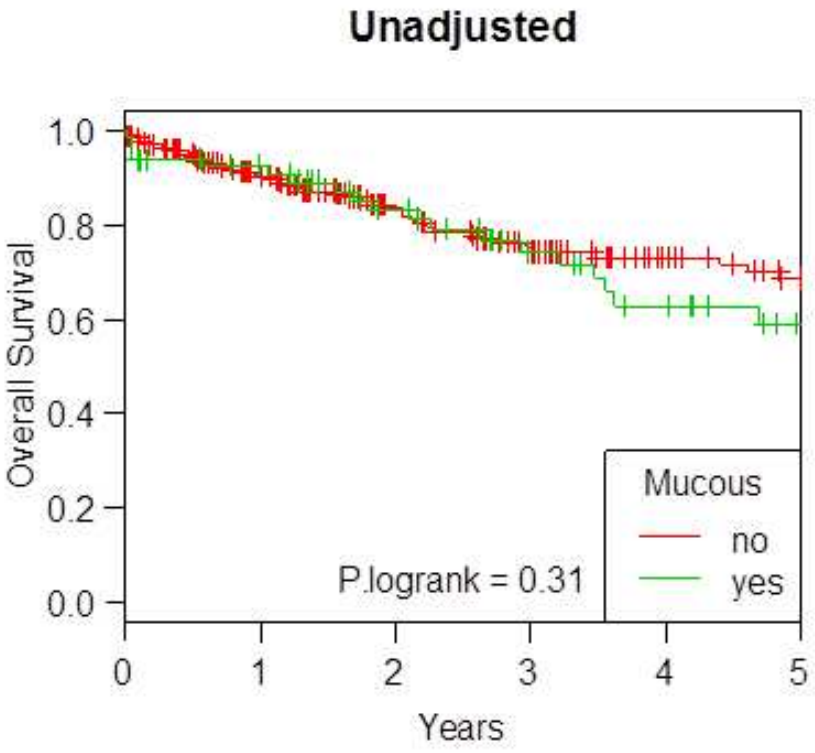

Figure 3.5: The two coloured curves represent distinct survival analyses based on mucinous and non-mucinous adenocarcinomas of the lung. The P.logrank test determines the presence of a difference between two curves. A p-value greater than 0.05 suggests no significant difference, while a p-value less than 0.05 indicates a significant difference between the curves. There is no difference in survival.

The distance between the curves is the relative hazard ratio and shows how the relative risk of death of mucinous is greater than in the case of a non-mucinous adenocarcinoma of the lung. The logarithm of this hazard ratio is 0.2678, illustrated by the coef in the table below. Because coef is a logarithm, so you can move exponentially, and therefore the hazard ratio of mucinous carcinoma is 1.31, or 30% higher than in the case of the non-mucinous. There is also a standard error and a P-value determining a 0-hypothesis, which says, that the coefficient or the exponent is 1. At 0.3, the 0-hypothesis is not rejected, so there is no difference between the groups. This is given in the following table.

n = 290, number of events = 67

(11 observations deleted due to missingness)

	coef	exp(coef)	se(coef)	z	Pr(>|z|)
Mucinous yes	0.2678	1.3071	0.2642	1.014	0.311

	exp(coef)	exp(-coef)	lower .95	upper .95
Mucinous yes	1.307	0.765	0.7787	2.194

Score (logrank) test = 1.03 on 1 df, p = 0.3094

Table 3.4: The hazard ratio for mucinous carcinoma, as indicated by the coefficient in the table, is 0.2678 in logarithmic form. By exponentiating the coefficient, we can interpret it as a hazard ratio of 1.31, meaning that mucinous carcinoma has a 30% higher hazard compared to non-mucinous carcinoma. The standard error and the corresponding p-value help determine whether the coefficient is statistically significant. In this case, with a value of 0.3, the 0-hypothesis is not rejected, indicating that there is no significant difference between the two groups. The details are presented in the table above.

Survival in clinically homogeous groups according to their TN stage: The corresponding hazard ratio of mucous carcinoma is 1.31 (95%CI: 0.77 to 2.20). The relative risk of 1 would be neutral. There was no difference between mucinous and non-mucinous carcinoma concerning survival after adjusting for clinical groups, however, the confidence interval was now 0.63 to 1.83.

Here are the results for the groups, as we do another comparison between mucinous and non-mucinous. We just have the variants group 1, group 2, and group 3. Examining the question if there is a percentage difference between lymph node metastasis versus no lymph node metastasis we compare the groups as seen in the following table. We compare if there is a marked difference in prognosis depending on the presence of LK metastases. So we compare mucinous and non-mucinous tumours within our three groups.

Mucinous						
Groups	no	yes				
T1/T2,No	133	34				
T1/T2,N1/N2	67	29				
T3/T4+N?	25	13				
In the higher groups there are fewer mucinous tumors (p=0.03, Wilcoxon test.)						
	coef	exp(coef)	se(coef)	z	Pr(>|z|)	
Groups T1/T2,N1/N2	0.58613	1.79702	0.28105	2.086	0.037*	
GroupsT3/T4+N?	1.35731	3.88574	0.34297	3.958	7.57e-05***	
Mucinous yes	0.07429	1.07712	0.27099	0.274	0.784	<P-Wert!
...						
Signf. Codes:	0***	0.001**	0.01*	0.05	0.1	1
	exp(coef)	exp(-coef)	lower .95	upper .95		
Groups T1/T2,N1/N2	1.797	0.5565	1.0359	3.117		
GroupsT3/T4+N?	3.886	0.2574	1.984	7.61		
Mucinous yes	1.077	0.9284	0.6333	1.832		

Table 3.5: The patients were divided into three groups based on the UICC TNM Classification. A p-value of 0.03 was obtained, indicating a significant difference between the lower and higher groups. Since the p-value is less than 0.05, it suggests that there is a statistically significant distinction between the higher and lower groups. The table above presents the hazard ratio for the three groups, comparing the mucinous and non-mucinous groups to determine if there is a significant difference between them. Once again, the 0-hypothesis is not rejected, indicating that there is no significant difference between the two groups. This suggests that there is no significant difference between mucinous and non-mucinous cases.

Statistically, we can see, that mucinous has no effect, and that is underpinned by several factors. Mucinous adenocarcinomas have no prognostic difference from non-

mucinous adenocarcinomas. They also behave biologically similar and the stage does not have any divergent effect in connection with either type. The LN-metastasis is equally common in both types and even if the patients are grouped with the LK-metastasis, in both groups the outcome is the same.

In the table above we see the relative risk for the mucinous adenocarcinoma within the T stage and in this case, we have another result. A relative risk of 1 is neutral. This means that no influence of mucinous adenocarcinomas can be detected. In the second table, we see a confidence interval of between 0.63 and 1.83. In summary, there is no difference between the groups and hence no correlation with survival.

There is no significant correlation between the mucinous and non-mucinous character of the tumours within the three groups.

There is no significant difference between the mucinous and non-mucinous character of the tumours within the three groups distinguished and illustrated graphically above.

Analysis of the mucinous and non-mucinous tumours within the T-Stage:
Now we analyse mucinous and non-mucinous tumours divided into 3 groups regarding their T-stage. The insight from the graphical representation is the same: If the tumour has a mucinous or non-mucinous character, does not influence the survival rate.

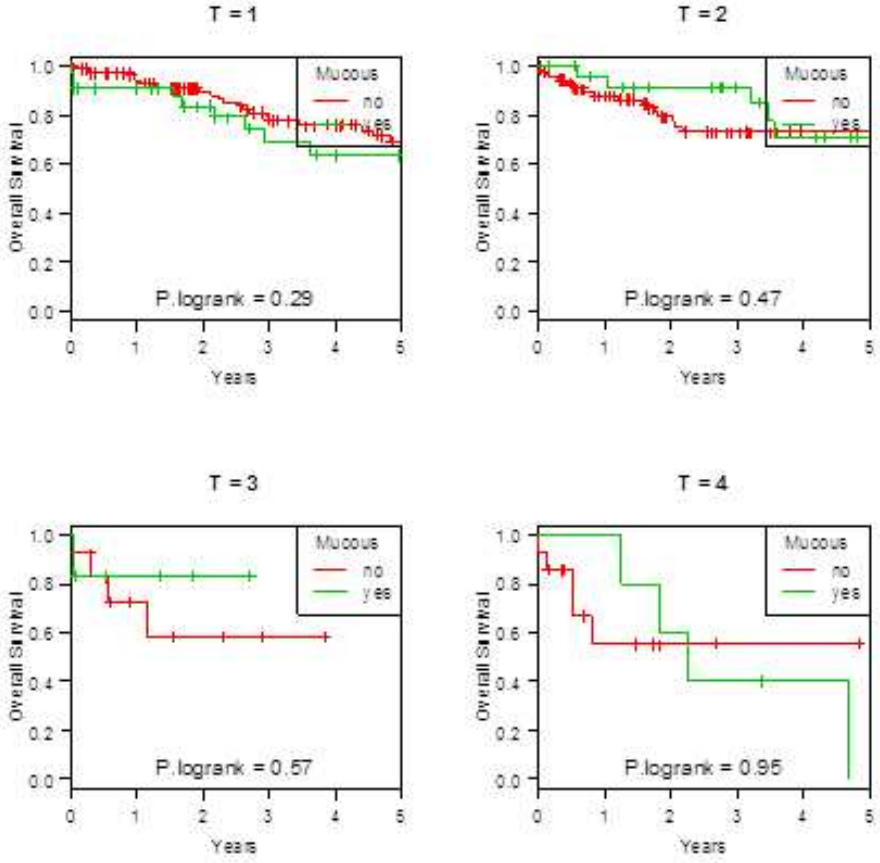

Figure 3.6: In each of the four diagrams, there are two coloured curves representing separate survival analyses for mucinous and non-mucinous adenocarcinomas of the lung, categorized by tumour size stages. The P.logrank test determines the presence of a difference between two curves. A p-value greater than 0.05 suggests no significant difference, while a p-value less than 0.05 indicates a significant difference between the curves. There is no difference in survival.

Test of the factor „mucous“ adjusted in the Cox model for T1 to T2: p=0.37. (LR-Criterium)

Test of the factor "mucinous" not adjusted in the Cox model: p=0.20. (LR-Criterium)

Test for connected T1 to T4 (ordinal) with mucous: p=43 (Spearman)

A Cox model was calculated as a test within the T-stages and the total P-value was

0.2. Hence, there is no significant difference and the presence of mucinous has no association with survival. Mucinous differentiation in adenocarcinomas does not influence survival. They behave biologically similar. The LN-metastasis is equally common in both types and even if the patients are grouped regarding the LN-metastasis, in both groups the outcome is the same.

In summary, there was no significant difference between mucinous and non-mucinous adenocarcinoma of the lung regarding the survival rate. The corresponding hazard ratio of mucinous carcinoma was 1.31 (95%CI: 0.77 to 2.20). A relative risk of 1 would be neutral. There was no difference between mucinous and non-mucinous carcinoma concerning survival even after adjusting for clinical groups, however, the confidence interval was now 0.63 to 1.83.

Among mucinous adenocarcinomas (ACs), the predominant patterns observed were acinar (43/71) followed by papillary (10/71).[10] Other patterns such as micropapillary, solid, and colloid types were present but in a few numbers (Figure 3.7 and Table 3.6).[10] Two cases displayed a predominant signet ring cell pattern, with one case showing a signet ring cell pattern throughout the entire tumour. Similar to non-mucinous ACs, most of the ACs exhibited secondary and even tertiary patterns (Table 3.6).

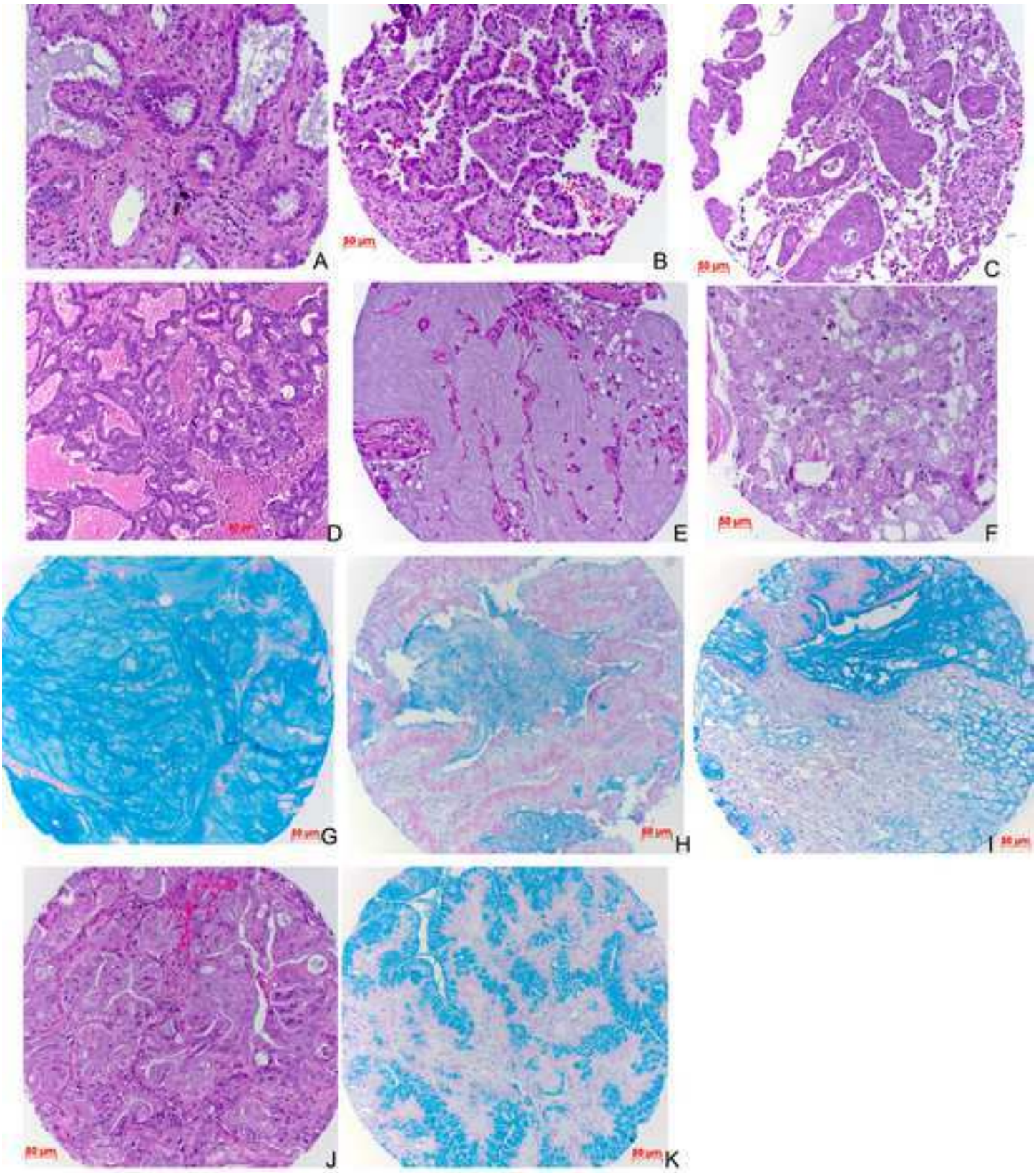
A
B
C
D
E
F
G
H
I
J
K

Figure 3.7: Pattern variations observed in mucinous adenocarcinomas include: A) Acinar, B) Papillary, C) Micropapillary, D) Cribriform, E) Colloid, F) Signet ring cell. Scale bar: 50 μm. Alcian blue stain demonstrates different patterns of mucin storage and secretion: G) Colloid (basolateral), H) Apical/luminal, I) Combined apical and basolateral. Goblet cell type is highlighted: J) Mucicarmine stain, K) Alcian blue stain. Scale bar: 50 μm.[10](Reproduced with permission from Springer Nature)

Predominant pattern	No of cases	Secondary component	No of cases
Acinar	43	Acinar	6
Papillary	10	Papillary	6
Micropapillary	4	Micropapillary	10
Cribriform	1	Cribriform	5
Solid	6	Solid	2
Lepidic	1	Lepidic	0
Signet ring cell	2	Signet ring cell	6
Colloid	4	Colloid	2

Table 3.6: The table illustrates the predominant and secondary patterns observed in mucinous adenocarcinomas.[10](Reproduced with permission from Springer Nature)

Type of mucin storage	Predominant pattern	Secondary pattern
Luminal mucin (acinar)	54	15
Extraluminal (basolateral) mucin	14	25
Goblet cell type	24	2

Table 3.7: Mucinous adenocarcinomas exhibit different types of mucin storage. The goblet cell variant consistently presents with luminal mucin secretion.[10](Reproduced with permission from Springer Nature)

Survival analysis:

We analyzed 330 adenocarcinoma patients including also mucinous for overall survival and correlated this with T and the N stages. There was a statistically significant difference between T-stages as well as N-stages as expected (log-rank test; P= 0.00067; Figure 3.2). For the N stages, we divided patients into groups according to their combined TN stage: Group 1 = T1 or T2 and N0 (T1/T2 and N0); Group 2 = T1 or T2 and N1 or N2 (T1/T2 and N1/N2); Group 3 = T3 or T4 and any N-group (T3/T4 and N$\geq$1). There was a statistically significant difference between N-stages (Figure 3.3; log-rank test; P= 0.000073).

Next, we looked for differences in survival of mucinous and non-mucinous ACs corrected for the stage: There was no difference between mucinous and non-mucinous adenocarcinoma of the lung, mucinous differentiation did not impact a worse outcome (Figures 3.6 and 3.8).

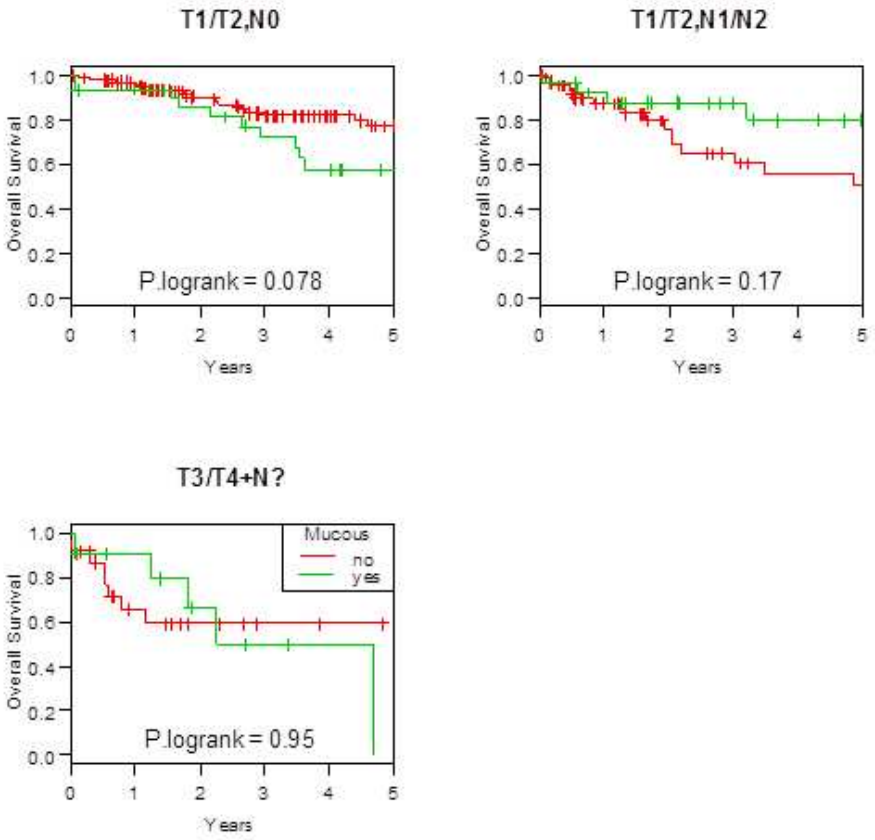

Figure 3.8: The two coloured curves in each of the three diagrams represent distinct survival analyses between mucinous and non-mucinous adenocarcinomas of the lung in three different groups according to the UICC TNM Classification of lung adenocarcinoma. The P.logrank test determines the presence of a difference between two curves. A p-value greater than 0.05 suggests no significant difference, while a p-value less than 0.05 indicates a significant difference between the curves. There is no difference in survival.

There was no significant difference between mucinous and non-mucinous adenocarcinomas within the three groups. There was neither a higher percentage of lymph node involvement in each stage in the mucinous or non-mucinous types nor any difference in survival. Even when tumours were compared for single T stage no difference was apparent (Figure 3.8).

Histochemistry and Immunohistochemistry:

Differences were observed between the staining results of full sections and tissue microarrays (TMAs). The variance between full section and TMA scores for *p14* and *p16* staining was less than 10%. Regarding *MUC1*, two cases displayed a higher percentage

and two cases exhibited a lower percentage compared to the TMA results. Notably, *TTF1* demonstrated the most significant difference, as full sections showed a higher percentage of positive tumour cells in 8 out of 20 cases. However, this disparity did not alter the results of the statistical analysis, with a p-value of 0.075 in the TMA group compared to 0.064 in the full sections.[10]

All cases exhibited reactivity for *CK7*, with the majority of cases showing expression in 100% of tumour cells. However, 9 cases displayed only focal positivity for *CK7*. In these 9 cases, *CK20* was co-expressed with *CK7*. None of the cases solely expressed *CK20*, but in most of them, *CK20* was detected in a small subset of tumour cells (less than 15%). Only 3 cases demonstrated a high proportion of *CK20*-positive cells (over 40%) (Figure 3.9a–b).

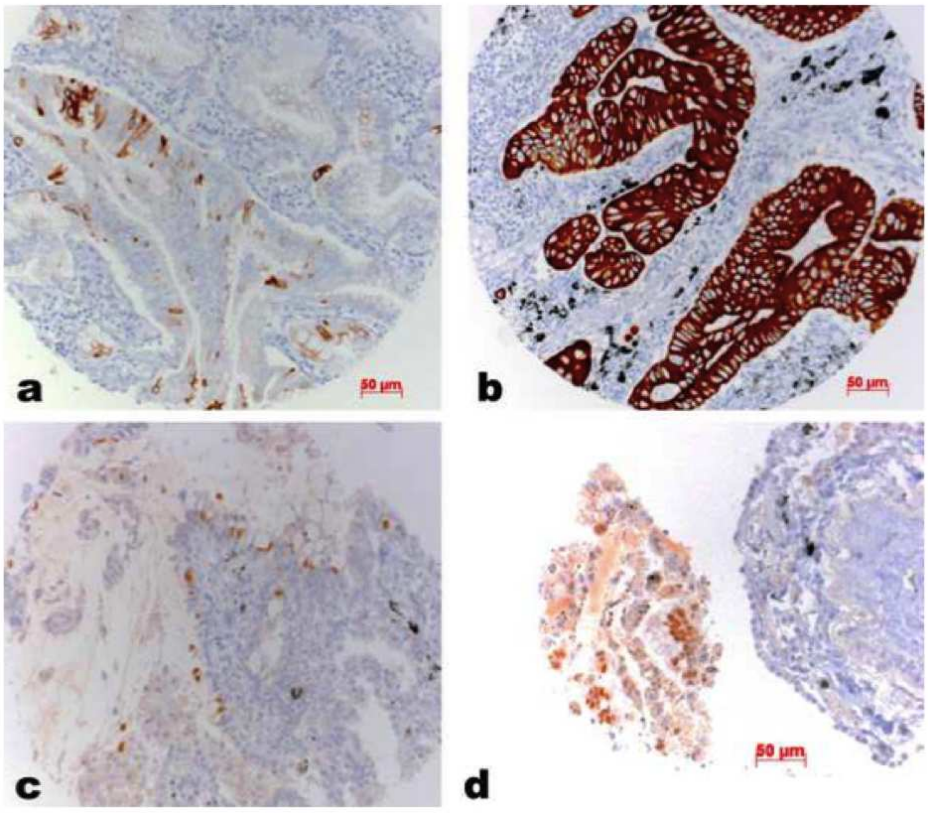

Figure 3.9: Immunohistochemical stains were performed for: a) *Cytokeratin 20*, b) *Cytokeratin 7*, c) *TTF1* in a case that co-expressed *CDX2*, and d) Single case that expressed *CDX2* but not *TTF1*. Scale bar: 50 μm.[10](Reproduced with permission from Springer Nature)

Among the 8 cases expressing *CDX2*, 7 showed expression in less than 10% of tumour cells, while one case exhibited expression in nearly 50% of tumour cells. Interestingly, this particular case was the only one that did not express *TTF1* (Figure 3.9 d). In contrast, the majority of other cases displayed predominant *TTF1* expression (Figure 3.9 c). TTF1 was observed in a few tumour cells containing mucin (Figure 3.9 c). Notably, co-expression of *CDX2* and *TTF1* in a single tumour cell was not observed. Furthermore, the expression of *CDX2* was consistently associated with *CK20* expression.[10]

Types of mucin secretion:
Mucin stains revealed diverse patterns within the adenocarcinomas, with luminal/apical mucin secretion observed predominantly in acinar adenocarcinoma types. A goblet cell pattern was identified in 24 cases, while all colloid adenocarcinomas exhibited extraluminal (basolateral) mucin secretion. However, a combination of luminal and extraluminal mucin staining (basolateral secretion) was observed in 33 cases. Notably, the presence of a goblet cell type of differentiation in mucinous adenocarcinomas was consistently associated with an apical/luminal type of mucin secretion (Figure 3.7). *MUC1* antibodies stained 39 cases, *MUC2* antibodies stained 7 cases, and *MUC5AC* antibodies stained 50 adenocarcinomas. In cases with goblet cell differentiation, either *MUC1* or *MUC5AC* was detected, and in a few instances, both *MUC* proteins were expressed.

Among the mucinous adenocarcinomas (ACs) stained for *p16INK4A*, 27 cases tested negative, while 19 cases exhibited a low H-score (below 40). In terms of *p14ARF* expression, 48 cases displayed moderate expression, 20 cases showed high expression, and 5 cases had a low *p14ARF* H-score (below 40) (Table 3.12). Unfortunately, the results of *p15* immunohistochemistry were not reproducible.[10]

KRAS mutation:
Out of a total of 71 cases, *KRAS* mutations were detected in 40 cases (56%). Mutations occurring at codon 12 were the most frequent. Specifically, 5 cases had mutations on codon 13, 5 cases had mutations on codon 61, and one case exhibited a double mutation (G12D and Q61R). No correlation was observed between *KRAS* mutation and the structural patterns. Furthermore, there was no association between *KRAS*

mutation and the stage of the disease, as they were equally present in both higher and lower stages. Among the 26 *KRAS* mutations detected in mucinous adenocarcinomas of the goblet cell type, 16 were codon 12 mutations, while only one was a codon 13 mutation. No significant differences were found between *KRAS* mutation status and adenocarcinomas with acinar, papillary, micropapillary, cribriform, and signet ring cell morphotypes. Additionally, *KRAS* mutations were found in all colloid adenocarcinomas (Tables 3.8 and 3.9)

G12V	10
G12D	7
G12C	9
G12S	2
G12R	1
G12L	1
G12A	1
G13C	4
G13D	1
Q61H	4
G12D+Q61R	1

Table 3.8: The different types of *KRAS* mutations and their respective frequencies.[10](Reproduced with permission from Springer Nature)

Acinar	WT	11
	mut	14
Papillary	WT	5
	mut	5
Micropapillary	WT	3
	mut	1
Solid	WT	3
	mut	1
cribriform	WT	1
	mut	1
Colloid	WT	0
	mut	4
Signet ring cell components	WT	9*
	mut	5*

Table 3.9: The correlation between *KRAS* mutations and the structural patterns of mucinous adenocarcinomas. The predominant patterns are provided, and only in cases featuring signet ring cells secondary and tertiary patterns are specified. WT = wild type *KRAS*; mut = mutated *KRAS*; * = 1 single case each characterized by a predominant or exclusive presence of signet ring cells.[10](Reproduced with permission from Springer Nature)

EML4ALK1 Rearrangement:

Among the samples analyzed, three cases exhibited positive immunohistochemistry results for *EML4ALK1* rearrangement. Additionally, two cases demonstrated positive findings through FISH analysis, indicating the presence of split signals in at least 30% of tumour cells. However, all cases tested negative when subjected to immunohistochemical staining for *ROS1*.[10]

Survival analysis:

We conducted a comprehensive analysis of 330 adenocarcinoma patients, including both mucinous and non-mucinous cases, to examine overall survival and its correlation with T and N stages. As expected, there was a statistically significant difference observed between T stages (I + II versus III + IV), as well as between N stages (N0 versus N1+N2) using the log-rank test (P=0.00067; Figures 3.2 and 3.3). Subsequently, we investigated the survival disparities between mucinous and non-mucinous adenocarcinomas while accounting for stages I-IV. The analysis revealed no significant difference in overall survival between the two types, nor were there any variations observed across different T stages. Mucinous differentiation did not contribute to a worse outcome (Figures 3.6 and 3.8). However, it is important to note that regardless of mucinous or non-mucinous status, clinically advanced-stage patients with lung adenocarcinoma experienced a significantly worse outcome (log-rank test; P=0.000073).[10]

To evaluate the N stages, we categorized patients into different groups based on their combined TN stage. Group 1 consisted of patients with T1 or T2 tumours and N0 status (T1/T2 and N0), Group 2 included patients with T1 or T2 tumours and N1 or N2 status (T1/T2 and N1/N2), and Group 3 encompassed patients with T3 or T4 tumours and any N group (T3/T4 and N$\geq$1). A statistically significant difference between the N stages was observed (Fig. 7) using the log-rank test (P=0.000073).

Upon investigating other variables potentially associated with mucinous differentiation, such as a higher incidence of lymph node metastasis in lower stages, it was found that mucinous types exhibited similar biological behavior. Lymph node (LN) metastasis was equally common in both types and even when patients were grouped based on their LN metastasis status, the outcomes were nearly identical. Stratifying the data according to N status did not reveal any discernible differences in outcome. Furthermore, there were no survival disparities observed between goblet cell and non-goblet cell types, as well as no distinctions based on the presence or absence of signet ring cells (Figure 3.10).[10]

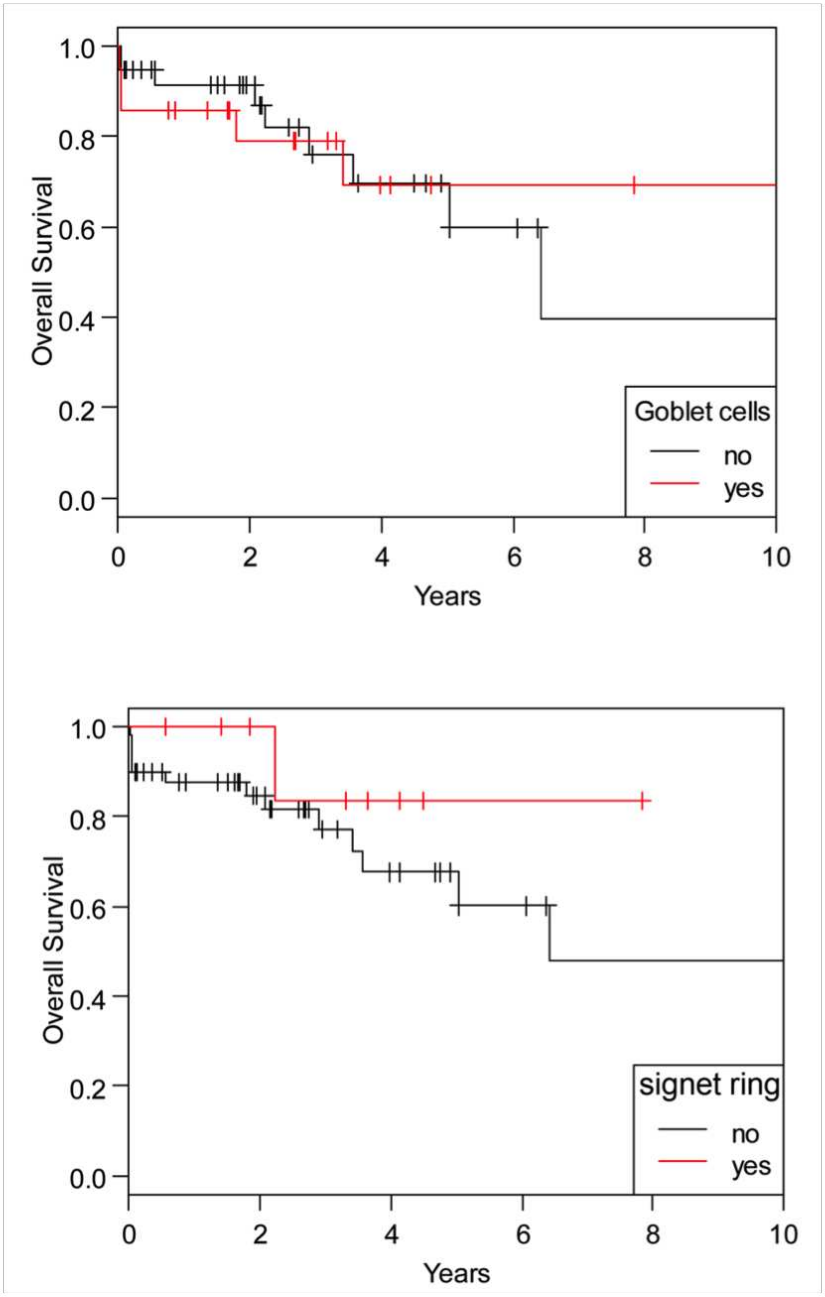

Figure 3.10: There was no observed difference in survival between goblet cell and non-goblet cell types of adenocarcinomas (upper panel), as well as between signet ring cell types and non-signet ring cell types of adenocarcinomas.

There was an observed but non-significant trend or difference indicating a stage-independent correlation between the loss of *p16INK4A* and a worse prognosis (Figure 3.11a). Conversely, there was no correlation between *p14ARF* and survival. Similarly,

there was a non-significant trend suggesting a correlation between high *TTF1* expression and improved survival (P=0.075, Figure 3.11b).[10]

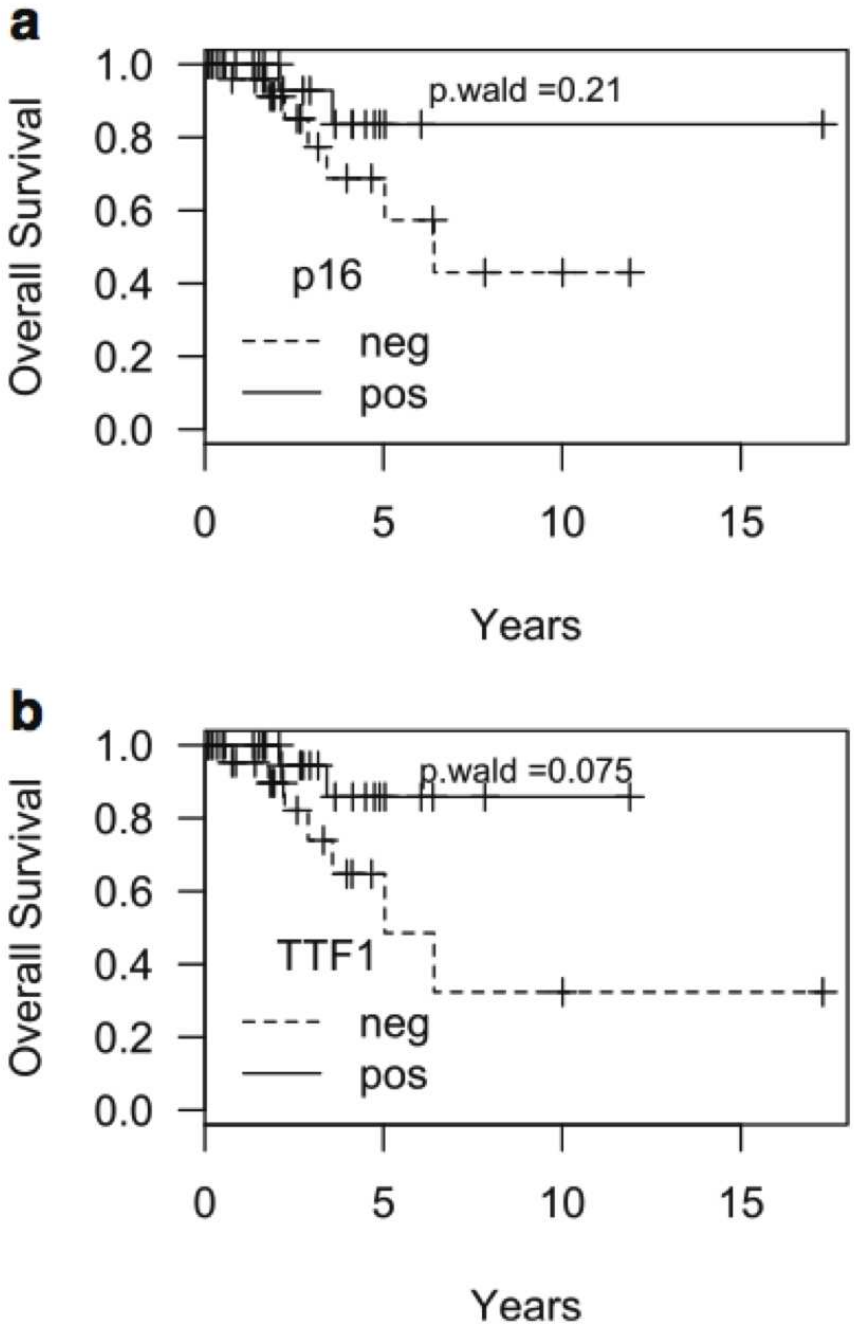

Figure 3.11: In the survival analysis comparing mucinous adenocarcinomas (ACs) based on *p16* loss versus positive staining (a), two separate analyses were conducted for *TTF1* (b): In the first analysis, *TTF1* cases were categorized as low/negative if they exhibited an intensity of 1+/2+ and involved less than 50% of tumour cells. In the second analysis, *TTF1* cases were classified as high/positive if they showed an intensity of 3+ in at least 50% of tumour cells.[10](Reproduced with permission from Springer Nature)

Table 3.10:

The patient's clinical data includes information on sex, age, and clinicopathological stage. Additionally, the mean and standard deviation of age are provided.[10](Reproduced with permission from Springer Nature)

Patient no	Sex	Age	Stage	Patient no	Sex	Age	Stage
1	f	59	IIIA	36	m	57	IA
2	f	58	IV[a]	37	m	74	IIB
3	f	65	IIB	38	m	55	IA
4	f	76	IIB	39	m	60	IB
5	f	60	IIA	40	m	48	IA
6	f	70	IA	41	m	64	IA
7	f	43	IIIA	42	m	58	IIA
8	f	43	IIIA	43	m	61	IIIA
9	f	43	IV[a]	44	m	56	IA
10	f	72	IB	45	m	72	IIIA
11	f	68	IA	46	m	68	IV[a]
12	f	70	IIA	47	m	65	IA
13	f	48	IIA	48	m	55	IV[a]
14	f	56	IA	49	m	64	IIIA
15	f	66	IIA	50	m	79	IA
16	f	81	IIIA	51	m	48	IIA
17	f	60	IIIA	52	m	81	IA
18	f	65	IIA	53	m	55	IB
19	f	73	IB	54	m	55	IIIA
20	f	64	IIA	55	m	72	IIA
21	f	53	IIA	56	m	52	IA
22	f	48	IIIB	57	m	69	IIB
23	f	59	IIIB	58	m	75	IIIA
24	f	71	IIB	59	m	68	IA
25	m	62	IA	60	m	70	IV[a]
26	m	66	IIIA	61	m	24	IA
27	m	56	IA	62	m	63	IIB
28	m	73	IIB	63	m	76	IIIA

29	m	60	IA	64	m	72	IIA
30	m	60	IIA	65	m	68	IIA
31	m	46	IIIA	66	m	48	IIA
32	m	66	IIA	67	m	63	IIIA
33	m	77	IIA	68	m	45	IIA
34	m	58	IA	69	m	80	IIB
35	m	55	IIA	70	m	84	IA
				71	m	73	IIA

f female, m male, [a] In these patients, staging was conducted intraoperatively using probatoria or mediastinal surgery, no lobectomy or pneumonectomy.

Table 3.11: (right below)

The antibodies used for immunohistochemistry and the FISH probes utilized are as follows.[10](Reproduced with permission from Springer Nature)

Antibody/clone	Company	dilution	Retrieval, visualization
CK 7 /OV-TL 12/30	Dako	1:100	Protease Typ XXIV, Dako Real, AEC
CK 20 /KS20.8	Dako	1:100	Protease Typ XXIV, Dako Real, AEC
TTF1 /SPT24	Ventana	ready to use	CC1, ultra view, DAB
CDX2 /EPR2764Y	Ventana	ready to use	CC1, ultra view, DAB
MUC1 /Ma695	Novocastra	1:100	Epitope Retrival, Dako Real, DAB
MUC2 /CC-8	Novocastra	1:50	Epitope Retrival, Dako Real, DAB
MUC5A /CLH5	Novocastra	1:100	TrisHcl+Urea, Dako Real, AEC
P14ARF /EPR3270	Abcam	1:15	CC1, ultra view, DAB
P15INK4B /EPR15057	Abcam	1:200	CC1, ultra view, DAB
P16INK4A /E6H4	MTM	ready to use	CC1, i view, DAB
ALK1 /D5F3	Ventana	ready to use	Optiview, DAB
ROS1 /D4D6	Cell Signaling	1:50	CC1, ultra view, fast red
FISH EML4ALK	Zytovision, TriCheck	ready to use	Zytovision FISH Implementation Kit
FISH ROS1	Zytovision Break Apart,	ready to use	Zytovision FISH Implementation Kit

Table 3.12:

Table : Patient data, diagnostic information, staging, type of mucin storage/secretion, KRAS mutational status, EML4ALK1 inversion, p14 and p16 values pooled from three different cores (TMA). Wild type KRAS is denoted as WT, IHC = immunohistochemistry, FISH = fluorescence in situ hybridization; mi = missing value; apical= columnar cell + apical; apical_goblet= goblet cell apical secretion; basolateral= stromal mucin; *In these particular patients, staging was performed intraoperatively by probatoria or mediastinal surgery; no lobectomy or pneumonectomy procedures.[10]

Diagnosis	mucin storage/secretion	MUC1	MUC2	MUC5AC	TTF1	sex	age	Grading/Staging	KRAS 12+13	KRAS 61	EML4ALK	p14	p16
signet ring	basolateral	5	0	285	0,0	m	1949	G2T1aN0 / IA	2,1%GGT>AGT G12S	WT		90,00	0,00
micropapillary signet ring	apical_basolateral	5	0	0	30,0	f	1949	G1T3N1 / IIIA	2,7%GGT>AGT G12S	WT	IHC++ FISH-	180,00	60,00
acinar colloid signet ring	apical_basolateral_goblet	18	8	2	0,0	m	1943	G2G3T4 / IIIA	GGC>GAC G13D 42,7%	WT		mi	mi
papillary micropapillary	apical_basolateral	126	0	150	0,0	m	1948	G1T1N1 / IIA	GGC>TGC G13C 54%	WT		80,00	6,67
acinar	apical	170	0	50	0,0	m	1932	G1G3T2N1 / IIB	GGC>TGC G13C 60%	WT		70,00	3,33
pred. papillary	apical_goblet	53	0	0	46,6	m	1943	G2T1 / IA	GGC>TGC G13C 65%	WT		150,00	76,67
pred.papillary	apical_basolateral	3	0	17	35,0	m	1943	G2T1N1 / IIA	GGC>TGC G13C 66%	WT		100,00	110,00
acinar papillary micropapillary	apical_basolateral_goblet	270	2	202	25,0	f	1951	G1G2T1aM1a / IV*	GGT>CGT G12R 32%	WT		72,50	80,00
acinar solid cribriform	apical_goblet	18	0	11	20,3	f	1945	G2T3N0V1L1 / IIB	GGT>CTT G12L 50%	WT		173,33	75,00
acinar	apical_goblet	11	0	195	12,8	f	1933	G1T3N0L1 / IIB	GGT>GAT G12D 16,7%	WT		60,00	26,67
acinar	apical_basolateral	106	0	0	66,6	f	1948	G2T2m / IIA	GGT>GAT G12D 16%	WT		106,67	160,00
colloid cribriform	apical_basolateral_goblet	170	73	123	1,7	m	1949	G2T2N2 / IIIA	GGT>GAT G12D 2,2%	CAA>CGA Q61R 10%		160,00	100,00
solid muzin AC with pleom.	apical_basolateral_goblet	240	90	70	4,5	m	1938	G2G3T1N1 / IIA	GGT>GAT G12D 3,6%	WT		70,00	0,00
acinar	apical_goblet	45	0	165	55,0	m	1932	G1T2aN1 / IIA	GGT>GAT G12D 31%	WT		110,00	0,00
acinar papillary signet ring	apical_goblet	16	0	300	8,3	f	1940	G1T1a / IA	GGT>GAT G12D 32%	WT		66,67	0,00
colloid	apical_basolateral	10	0	0	0,0	m	1948	G1T1 / IA	GGT>GAT G12D 8,4%	WT		40,00	0,00
acinar micropapillary solid signet ring	apical	280	0	40	23,3	m	1953	G2G3T1bN1 / IIA	GGT>GCT G12A 38%	WT		67,50	33,33
acinar	apical_ex	65	0	260	7,3	m	1955	G1T1aN0 / IA	GGT>GTT G12V 10,6%	WT		80,00	13,33
solid mucinous AC		10	0	300	1,7	m	1928	G1T2 / IIB	GGT>GTT G12V 10%	WT		60,00	0,00
mucinous papillary	apical_basolateral	20	0	300	0,0	m	1944	G2T1mN0 / IA	GGT>GTT G12V 12%	WT		40,00	0,00
acinar	apical_basolateral	mi	0	0	5,0	f	1954	G2T4 / IIIA	GGT>GTT G12V 14%	WT		100,00	0,00

acinar	apical_basolateral	35	0	150	70,0	f	1954	G2G3T4mN1 / IIIA	GGT>GTT G12V 15%	WT		75,00	0,00
acinar	apical_basolateral	mi	mi	80	23,3	f	1954	G1T4mN1M1a / IV*	GGT>GTT G12V 20%	WT		80,00	0,00
acinar mucinous	apical_goblet	0	0	250	8,3	f	1938	G1T2N0 / IB	GGT>GTT G12V 25%	WT		70,00	33,33
predom. Lepid. (muzin)	apical_basolateral_goblet	13	0	300	21,6	m	1930	G1T2N0 / IB	GGT>GTT G12V 29%	WT		80,00	6,67
solid muzin.AC	apical_basolateral	5	0	300	3,0	f	1951	G1T1a / IA	GGT>GTT G12V 5%	WT		46,67	10,00
acinar	apical_goblet	16	0	86	5,0	f	1942	G1T4N0L1 / IIIA	GGT>GTT G12V 9%	WT		33,33	10,00
acinar	apical_basolateral	270	0	13	0,0	m	1936	G1G2T1N0 / IA	GGT>TGT G12C 13%	WT		70,00	13,33
acinar	apical_basolateral_goblet	56	0	96	53,3	m	1961	G2T1aN0 / IA	GGT>TGT G12C 13%	WT		63,33	73,33
mucinous acinär	apical_goblet	mi	0	10	0,0	f	1937	G2T2N1 / IIA	GGT>TGT G12C 2,4%	WT		10,00	0,00
u acinar-cribriform	apical_basolateral_goblet	270	0	60	1,7	m	1956	G1G2T2N1 / IIA	GGT>TGT G12C 23%	WT		33,33	133,33
papillary micropapillary	apical_goblet	260	0	195	0,0	m	1943	G3T2N2 / IIIA	GGT>TGT G12C 24%	WT		55,00	6,67
mucinous colloid	apical	0	80	0	40,0	m	1945	G1T1N0 / IA	GGT>TGT G12C 3,5%	WT		50,00	180,00
acinar	apical	240	0	10	6,6	m	1953	G1T3N2 / IIIA	GGT>TGT G12C 42%	WT		100,00	0,00
acinar	apical	mi	mi	mi	mi	m	1935	G1T4mN2M1b / IV*	GGT>TGT G12C 71,3%	WT		mi	mi
colloid	apical_goblet	40	0	10	35,0	m	1942	G1T1aN0 / IA	GGT>TGT G12C 9%	WT		50,00	20,00
mucinous signet ring	basolateral	mi	mi	mi	mi	m	1942	G2T4mN2M1b / IV*	WT	CAA>CAC Q61H 20%		mi	mi
solid spindel	apical_basolateral	40	0	0	40,0	f	1956	G1T1a / IA	WT	CAA>CAC Q61H 35%		90,00	0,00
solid pleomorph 10% spindelz.	apical_basolateral	60	0	0	0,0	m	1955	G3T3N1 / IIIA	WT	CAA>CAC Q61H 53%		35,00	0,00
acinar papillary	apical_basolateral	0	0	300	15,0	m	1937	G2T1N0 / IA	WT	CAA>CAT Q61H 13,5%		90,00	0,00
acinar	apical	5	0	0	83,3	f	1933	G2T2N1 / IIA	WT	WT		170,00	13,33
acinar	apical_basolateral	5	0	0	0,0	m	1940	G1G2T2N1 / IIA	WT	WT		80,00	0,00
acinar solid	apical_basolateral	200	0	0	70,0	m	1959	G2G3T1N0L1V1 /IA	WT	WT		53,33	210,00
micropapillary signet ring	apical_basolateral	53	3	4	38,0	f	1926	G2G3T4N1 / IIIA	WT	WT		133,33	250,00
micropapillary	apical_basolateral	mi	mi	mi	38,0	f	1926	G2G3T4N1 / IIIA	WT	WT		93,33	180,00
acinar papillary micropapillary	apical_goblet	270	0	16	63,3	m	1947	G1T2N0L1 / IB	WT	WT		83,33	250,00

predom.papillary, acinar	apical	11	0	45	0,0	m	1955	G2T3N2 / IIIA	WT	WT		113,33	26,67
papillary u mikropap Ly-meta,	apical	5	0	60	0,0	m	1955	G2T2aN1V1L1 / IIA	WT	WT		105,00	0,00
cribriform(acinar	apical_basolateral	115	0	15	0,0	m	1931	G2G3T1N0 / IA	WT	WT		45,00	180,00
acinar papillary	apical_basolateral	125	0	13	0,0	m	1957	G2T2bN1 / IIB	WT	WT		96,67	0,00
acinar signet ring	apical_goblet	16	0	0	46,6	f	1935	G1T1N1 / IIA	WT	WT		82,50	13,33
acinar	apical_basolateral	226	0	206	0,0	m	1928	G2T1N2 / IIIA	WT	WT		80,00	93,33
acinar	apical_goblet	53	0	20	0,0	m	1934	G1T1aN0 / IA	WT	WT		33,33	0,00
acinar	apical_basolateral	290	0	10	3,3	m	1941	G2T4mN2M1b / IV*	WT	WT		100,00	3,33
mucinous micropapillary	apical_basolateral_goblet	60	0	25	0,0	m	1927	G1T1N0 / IA	WT	WT		190,00	0,00
acinar micropapillary	apical_basolateral	20	5	210	5,3	m	1984	G2T2N1 / IIB	WT	WT		120,00	0,00
acinar cribriform	apical	5	0	0	60,0	m	1945	G1G2T2N2L1V1 / IIIA	WT	WT	IHC+++ FISH+	115,00	135,00
papillary acinar	apical_goblet	150	0	106	0,0	m	1932	G2G3T2mN1 / IIA	WT	WT		53,33	30,00
acinar cribriform	apical	30	0	33	65,0	m	1936	G2T1aN1 / IIA	WT	WT		140,00	46,67
solid signet ring	apical_goblet	260	0	220	23,3	f	1940	G2T2N0 / IB	WT	WT		76,67	153,00
acinar	apical	0	0	0	10,0	f	1936	G2T1 / IA	WT	WT		60,00	0,00
acinar	apical_basolateral	56	0	0	73,3	f	1937	G2G3T2N1 / IIA	WT	WT		90,00	73,33
acinar micropapillary	apical_goblet	150	0	80	50,0	m	1946	G1T1N1 / IIA	WT	WT	IHC+++ FISH+	115,00	20,00
acinar micropapillary signet ring	apical_goblet	223	0	130	2,5	f	1961	G1G3T4N3L1 / IIIB	WT	WT		77,50	0,00
acinar papillary signet ring	apical_basolateral	200	0	135	0,0	f	1961	G1G3T4N3 / IIIB	WT	WT		60,00	0,00
acinar	apical	6	0	96	1,6	m	1950	G2N3 / IIIA	WT	WT		50,00	0,00
acinar micropapillary	apical	265	0	22	42,5	m	1948	G1G3T2aN1 / IIA	WT	WT		97,50	0,00
acinar micropapillary signet ring	apical_basolateral	300	0	4	0,0	m	1966	G3T3N0 / IIB	WT	WT		95,00	23,33
papillary acinar solid	apical_basolateral	120	0	0	75,0	f	1931	G3T3N0L1V1 / IIB	WT	WT		90,00	260,00
acinar	apical_ex_goblet	6	0	0	66,6	m	1940	G2T1aN0 / IA	WT	WT		50,00	260,00
papillary acinar micropapillary	apical	28	0	21	63,3	m	1927	G3T1bN1 / IIA	WT	WT		103,33	0,00

3.4 Discussion

It is typical for T-stage 1, that if the patients are disease-free for more than 6 or 7 years, they are basically healed and have a natural life span. This applies to approximately 40% of patients with T-stage 1 in our study.

Mucinous adenocarcinomas make up approximately one-third of all lung adenocarcinomas. The differentiation into a mucinous phenotype appears to occur early in carcinogenesis, suggesting that the precursor lesion already possesses this differentiation.[29] Previous studies have frequently reported that mucinous adenocarcinomas of the lung have a poorer prognosis compared to non-mucinous ones. However, only a few studies have directly compared the behavior and overall survival of mucinous and non-mucinous adenocarcinomas. Adding to the complexity, there is some confusion within this category, particularly regarding solid adenocarcinomas, which are classified as non-mucinous despite requiring at least 10% of tumour cells to exhibit mucinous differentiation.[10]

The perception that mucinous adenocarcinomas confer a worse prognosis than non-mucinous counterparts has been widely propagated without thorough re-examination.[33] One study by Carretta et al., which included 49 patients, 42 of whom underwent complete resection with histological examination, showed mucinous differentiation in 13 patients. They found a worse outcome in patients with a mucinous pattern in stage IA and IB, but the sample size was small and included a mix of invasive and in-situ adenocarcinomas (previously known as bronchioloalveolar carcinomas).[34] In another study by Riquet et al., a comparison was made between solid adenocarcinomas with and without mucin components. The study included 1139 resected lung cancer patients, of whom 565 were classified as LUAC (lung adenocarcinoma), with 239 identified as solid LUAC with a mucin component. It was observed that solid adenocarcinomas with mucin production had a worse prognosis. However, the authors' comparison of "solid adenocarcinomas without mucin production" is not valid since solid adenocarcinomas are defined as having mucin-producing cells. Therefore, their solid carcinomas may belong to a completely different entity, possibly large cell carcinomas. This study highlights the confusion created by the WHO classification. Although it may seem logical to

differentiate between solid adenocarcinomas with and without mucin production, solid adenocarcinomas themselves have a worse prognosis compared to well-differentiated adenocarcinomas, regardless of mucin production.[86][87][10]

Interestingly, when examining mucinous-type bronchioloalveolar adenocarcinomas (now classified as in-situ adenocarcinomas, AIS), a favorable course is observed compared to invasive adenocarcinoma. This further emphasizes the need to consider histological subtypes and classifications when assessing prognosis and outcomes in lung adenocarcinomas.[88]

When we compared mucinous and non-mucinous adenocarcinomas, taking into account the stage and lymph node involvement, we observed similar behavior between the two groups. Additionally, in our extensive series of mucinous adenocarcinomas, we did not identify any significant differences in behavior when comparing goblet cell types to non-goblet cell types. Although we encountered a few cases of mucinous adenocarcinomas with signet ring cell components, including two cases with dominant or pure signet ring cell carcinomas, we did not observe a noticeable difference in behavior when these cases were adjusted for stage. This finding contradicts a published study that reported a prognosis based on only two cases of signet ring cell carcinomas, which we consider too small of a sample size to draw any definitive conclusions.[88] It is important to note that even our limited number of cases is insufficient to provide a final conclusion regarding the behavior of the signet ring cell subtype of adenocarcinomas.[10]

Upon examining the immunohistochemical profile, we observed that the majority of mucinous adenocarcinomas exhibited *TTF1* expression, often in conjunction with *CK7* expression. A minority of cases coexpressed *CK20*, and a few also expressed *CDX2*, which is typically used as a marker for intestinal adenocarcinomas that metastasize to the lung. Notably, with the exception of one case, all the tumours showed subsets of tumour cells coexpressing *TTF1*. This suggests the presence of two distinct clones: one expressing *TTF1* and *CK7*, and another expressing *CDX2* and *CK20*. Previous studies on this subject had limited case numbers: 13 patients [88], 19 patients [89], and 19 patients [90], respectively. When further subclassifying these already small sets,

the sample size for the subtypes of mucinous adenocarcinomas, including AIS cases, became even smaller.[88] [89] In contrast to the study by Shah et al.[90], all of our mucinous adenocarcinoma cases demonstrated *CK7* expression, at least in a portion of the tumour cells. We observed a correlation between *CK7* expression and *TTF1* expression, whereas *CK20* expression was only detected in a minority of mucinous adenocarcinomas, and in some cases, it was associated with *CDX2*, aligning with the findings of the study by Yatabe et al.[89] Several studies have also investigated the expression of *CK7* and *CK20* and reported similar findings to our study.[88][90][91][92][10]

When classifying mucinous adenocarcinomas based on their mucin-type, we consistently observed the presence of acidic mucins in all our cases, as demonstrated by positive staining with mucicarmine and Alcian blue at pH 2.5. We stained our cases for *MUC* proteins as done in previous reports. Rossi et al. and Yatabe et al. have shown *MUC2* expression in goblet cell types and *MUC5AC* expression in other variants. Interestingly, conflicting data exist in the literature: *MUC1* expression has been found in adenocarcinomas lacking goblet cell morphology[93], while Kunii confirmed the expression of *MUC2* and *MUC5AC*.[94]. In our study *MUC1* antibodies stained 39 cases, *MUC2* antibodies stained 7 cases, and *MUC5AC* antibodies stained 50 cases. In cases with goblet cell differentiation, either *MUC1* or *MUC5AC* was detected, and in a few instances, both *MUC* proteins were expressed. In the study by Kunii et al., they observed the expression of *MUC2* and *MUC5AC*, along with *HNF4alpha*, in cases negative for *TTF1*. It is worth noting that the number of *TTF1*-negative mucinous adenocarcinomas in their study was relatively high, which may suggest potential differences between the Caucasian and Asian populations and warrants further investigation.[94][10]

Investigations have revealed that the function of *TTF1* varies depending on the tumour type. In non-mucinous adenocarcinomas, *TTF1* plays a role in upregulating the expression of *downstream surfactant genes* and is linked to the *NapsinA gene*.[95] *TTF1*, also known as *NKX2-1*, is a transcription factor responsible for the differentiation of lung epithelial cells, preventing gastric differentiation. Downregulation of *TTF1* in lung adenocarcinomas can lead to a poorly differentiated tumour type. Ad-

ditionally, research by Mesquita has shown that *TTF1* is involved in the expression of *MUC2* in *CDX2*-positive mucinous adenocarcinomas, where it acts as a transcriptional regulator.[96]

In our series of mucinous adenocarcinomas, *KRAS* mutation was the most frequently observed alteration, accounting for 56% of cases. This prevalence significantly contrasts with the 20-25% positivity seen in non-mucinous variants but aligns with previous studies.[89][93][97][98][99][100][101] Even in cases of mucinous adenocarcinoma in situ (AIS), *KRAS* mutation was predominant.[102] We detected *KRAS* mutations in 40 out of 71 cases. The majority of these mutations were found in codon 12. Mutations in codon 13 were present in 5 cases, and mutations in codon 61 were found in another 5 cases, with one case exhibiting a double mutation, G12D and Q61R. *KRAS* mutation status did not differ among lung adenocarcinomas (LUAC) with acinar, papillary, micropapillary, cribriform, goblet cell, or signet ring cell variants, nor were there any correlations with disease stage. However, all cases of colloid adenocarcinoma were KRAS mutants. This finding contrasts with studies by Yatabe and Mashima, which reported no KRAS oncogene mutations in cases with goblet cell morphology. Further investigations are needed to determine whether these discrepancies are related to differences in the ethnic backgrounds of the cases studied. In cases with signet ring cell components, we observed both wild-type and mutated KRAS cases, while all mucinous adenocarcinomas with colloid components were KRAS mutated. The timing of KRAS mutations, whether they occur early or late in tumourigenesis, remains a topic of debate.[103][104][105][106] The high proportion of *KRAS* mutations in mucinous adenocarcinomas offers hope for targeted therapies aimed at downstream kinases of the RAS pathway, once available.[107][108] However, further analysis of downstream pathways is necessary to identify specific signaling cascades that might be targeted, including the MAPK-ERK pathway, RAL pathway, PI3K pathway, or PLCϵ pathway.[10]

In addition to the factors mentioned, there are other potential contributors to the development of mucinous adenocarcinomas. Inflammatory cytokines have been demonstrated to influence the proliferation and transformation of goblet cells, suggesting their involvement in early genetic abnormalities that give rise to precursor lesions

with mucinous differentiation.[59][30][109][110][111][112] Studies have to explore these mechanisms, utilizing mouse models to gain a deeper understanding of the underlying processes.[10]

Out of 74 mucinous lung adenocarcinomas, only 2 cases exhibited *EML4-ALK* rearrangements, both of which had wild-type *KRAS* sequences. The positive cases were found to have acinar/cribriform and acinar/micropapillary patterns upon retrospective review. A third case showed high *ALK* protein expression but did not have an *EML4-ALK* rearrangement; instead, it had a *KRAS* codon 12 mutation. No *ROS1* rearrangements were detected in any of our cases.
In our series of mucinous adenocarcinomas, more than 40% of the cases did not show any identified driver mutation. To gain more insights, we conducted next-generation sequencing analysis on four of these cases using a comprehensive cancer panel, which included *LKB1/STK11*. None of these cases exhibited a positive *LKB1* mutation. Further analysis is required for cases that tested negative for *KRAS* mutations.
It is important to note that among the cases with wild-type *KRAS*, other mutations may still be discovered. Therefore, addressing this question effectively necessitates a new investigation using specifically designed cancer panels.[10]

Loss or reduced expression of *p16INK4A* and *p14ARF* was observed in 44 cases. The absence or loss of *p16INK4A* was linked to poorer overall survival, although this finding was not statistically significant. In contrast, *p14ARF* loss was not associated with survival outcomes. Both *p14ARF* and *p16INK4A* are checkpoint control proteins during mitosis, directing cells with defective DNA either towards repair or apoptosis. *p14ARF* functions at the G1 transition, while *p16INK4A* collaborates with p53 at the G2 checkpoint. Given that mucinous LUAC patients are predominantly smokers and have frequent p53 mutations, further research into the relationship between p53 mutations and loss of *p16INK4A* function is necessary.

Similar to other histological types of adenocarcinomas, the presence of mucinous differentiation in lung adenocarcinomas does not currently influence therapy or prognosis. However, mucinous differentiation may have additional effects that are yet to be

fully understood, highlighting the need for further research. The subtypes of adenocarcinoma, including mucinous differentiation, do not independently impact prognosis. Therefore, there are likely other factors contributing to the differentiation of lung adenocarcinomas that are responsible for prognosis variations.[10]

Although we had a large number of patients for the study, the sample size was limited for some individual examinations and not representative enough to draw definitive conclusions. While Tissue Microarray (TMA) staining is a valuable technique for analyzing multiple samples simultaneously, it has limitations. It may not fully reveal the heterogeneity in morphology or staining because it involves taking small core samples from a larger tissue specimen, which can lead to sampling bias. If the core is not representative of the overall tumor, it may result in inaccurate conclusions about the presence or absence of specific markers. Additionally, TMA preparation is technically challenging, requiring precise alignment and cutting, as well as addressing pronounced fixation or processing issues. Misalignment or damage to the cores can compromise the quality of staining or the results, potentially leading to false-negative or false-positive outcomes.[113]

3.5 Conclusion

Within pulmonary adenocarcinomas, mucinous types constitute about 25-30% of cases. Mucinous adenocarcinomas are considered to be more aggressive than non-mucinous adenocarcinomas. All mucinous adenocarcinomas are not subtyped with the exception of colloid and enteric adenocarcinomas. 71 mucinous LUAC were analyzed, including colloid adenocarcinomas, for their predominant and secondary patterns, their different form of mucin storage and release, their expression of *CK7* and *20*, *TTF1* and *CDX2*, and *MUC1*, *2* and *5AC* proteins, their expression of *p14*, and *p16* proteins, their possible rearrangements for *EML4ALK* and *ROS1*, as well as their *KRAS* mutational status and correlated this with survival. We selected 259 non-mucinous adenocarcinomas for comparison.[10]

A statistically significant distinction was observed between the T- (log-rank test; P= 0.00067) and N-stages (log-rank test; P= 0.000073). The corresponding hazard ratio of mucinous carcinoma was 1.31 (95%CI: 0.77 to 2.20). A relative risk value of 1 indicates a neutral outcome. After adjusting for clinical groups, there was no discernible distinction in survival between mucinous and non-mucous carcinoma. However, the confidence interval now ranged from 0.63 to 1.83.

Overall survival of mucinous adenocarcinomas corrected for T and N stage was not different from their non-mucinous counterpart. The acinar pattern was most frequently seen. Neither pattern, nor type of mucin storage, such as luminal, extracellular, or goblet cell type had any influence on survival.[10]

In our study, we could not see any difference in survival rates between mucinous or non-mucinous adenocarcinomas of the lung. This is in contrast to the literature that states that the mucinous have a worse prognosis than the non-mucinous tumours.
Several adenocarcinomas were positive for *CK20*, all of them except one expressed *TTF1* either strongly or at least focally, eight coexpressed *CDX2* focally. Most mucinous adenocarcinomas expressed either *MUC1* or *MUC5AC* proteins, rarely *MUC2*. Some cases coexpressed both or all three. In mucinous adenocarcinomas, a loss of *p16* expression correlated with a worse outcome. Mucinous adenocarcinomas exhibit mutation of *KRAS* oncogene in 56%. *KRAS* mutational status was neither correlated with architectural pattern nor survival. Most frequent were codon 12 mutations, one case presented with double *KRAS* mutations in codon 12 and 61.
The high proportion of KRAS mutations could be important for targeted therapy, particularly through the inhibition of downstream kinases in the RAS pathway, such as those involved in the MAPK-ERK, RAL, or PI3K pathways.[10]
Goblet cell variants of mucinous adenocarcinomas presented predominantly with codon 12 mutations. All colloid adenocarcinomas had *KRAS* mutation. Two cases had EML4 and ALK1 rearranged, *ROS1* rearrangement was not found. Mucinous adenocarcinomas behave similarly to non-mucinous variants. TNM stage is the most important factor followed by *p16* loss predicting overall survival. In our study, we could not see any difference in survival rates between mucinous or non-mucinous adenocarcinomas

of the lung. This is in contrast to the literature that states that the mucinous have a worse prognosis than the non-mucinous tumours. TNM is the most important factor, which is predicting overall survival. This is followed by the *p16* loss.[10]
As I previously mentioned, the sample size was limited for some individual examinations. Therefore, I suggest collecting additional patient data and increasing the sample size, possibly through collaboration with other centers. This would enhance the study's statistical power and allow for more definitive conclusions. We also recommend supplementing TMAs with whole-slide imaging or other techniques to enhance the assessment of tumor heterogeneity. Additionally, I propose a statistical evaluation of clinical data, such as the potential stronger association between lung adenocarcinoma and malignant pleural effusions, which could be better assessed with a larger dataset. Furthermore, I recommend conducting a similar study on squamous cell lung cancer to facilitate a comparison between the two cancer types.

4Supplementary Materials

Table 4.1:

The predominant pattern observed in mucinous adenocarcinomas was analyzed in terms of gender and age distribution, as well as the presence of specific mutations in KRAS, rearrangements in ALK1, and ROS1. "WT" = wild type, and mutations are presented as nucleotide mutation and subsequent codon alterations. The status of EML4ALK1 and ROS1 was determined using immunohistochemical scoring (+ to +++), and FISH (+) analysis. Pathological staging was conducted based on surgical findings, with all patients clinically classified as M0. * Cases without sufficient clinical data were excluded from survival analysis, while ** cases with insufficient tissue were excluded from molecular analysis.[10](Reproduced with permission from Springer Nature)

predominant pattern	gender	age	pGTN stage	KRAS Codon 12+13	KRAS codon 61	EML4ALK
acinar	f	79	G2T2N1	WT	WT	
acinar	m	66	G1G2T2N1	WT	WT	
acinar	m	70	G1G2T1N0	GGT>TGT G12C 13%	WT	
colloid	m	61	G1T1N0	GGT>TGT G12C 3,5%	WT	
acinar	m	72	G1M1b	GGT>TGT G12C 71,3%	WT	
acinar	m	48	G2G3T1N0L1V1	WT	WT	
micropapillary	f	81	G2G3T4N1	WT	WT	
micropapillary	f	81	G2G3T4N1	WT	WT	
acinar	m	60	G1T2N0L1	WT	WT	
signet ring	m	65	G2M1b	WT	CAA>CAC Q61H 20%	
acinar	f	71	G1T2N0	GGT>GTT G12V 25%	WT	
acinar	*	*		GGT>GAT G12D 46%	WT	
papillary	m	55	G2T3N2	WT	WT	
solid	f	60	G1T1a	GGT>GTT G12V 5%	WT	
lepidic	m	72	G1T2N0	GGT>GTT G12V 29%	WT	
papillary	m	55	G2T2aN1V1L1	WT	WT	
acinar	m	48	G1G2T2N1	GGT>TGT G12C 23%	WT	
solid	m	56	G3T3N1	WT	CAA>CAC Q61H 53%	
acinar	f	82	G2G3	WT	WT	
solid	m	74	G1T2	GGT>GTT G12V 10%	WT	
solid	m	66	G2G3T1N1	GGT>GAT G12D 3,6%	WT	
papillary	m	60	G2T1	GGC>TGC G13C 65%	WT	
papillary	m	60	G2T1N1	GGC>TGC G13C 66%	WT	
cribriform	m	72	G2G3T1N0	WT	WT	
acinär	f	64	G2T2N1	GGT>TGT G12C 2,4%	WT	
acinar	m	52	G2T2bN1	WT	WT	

acinar	*	*		GGT>TGT G12C 10,1%	WT	
papillary	m	56	G1T1N1	GGC>TGC G13C 54%	WT	
acinar	f	69	G1T1N1	WT	WT	
acinar	m	73	G1G3T2N1	GGC>TGC G13C 60%	WT	
acinar	f	43	G2T4	GGT>GTT G12V 14%	WT	
acinar	m	65	G2T1N2	WT	WT	
colloid	m	46	G2T2N2	GGT>GAT G12D 2,2%	CAA>CGA Q61R 10%	
acinar	m	48	G2T1aN0	GGT>TGT G12C 13%	WT	
acinar	m	75	G1T1aN0	WT	WT	
acinar	f	43	G2G3T4mN1	GGT>GTT G12V 15%	WT	
acinar	f	43	G1M1a	GGT>GTT G12V 20%	WT	
acinar	f	58	G1G2	GGT>CGT G12R 32%	WT	
acinar	m	68	G2M1b	WT	WT	
micropapillary	m	70	G1T1N0	WT	WT	
colloid	m	58	G1T1	GGT>GAT G12D 8,4%	WT	
acinar	m	56	G1T3N2	GGT>TGT G12C 42%	WT	
acinar	m	24	G2T2N1	WT	WT	
acinar	m	63	G1G2T2N2L1V1	WT	WT	+++/+
micropapillary	f	59	G1T3N1	2,7%GGT>AGT G12S	WT	++ /-
papillary	m	76	G2G3T2mN1	WT	WT	
acinar	m	72	G2T1aN1	WT	WT	
solid	f	68	G2T2N0	WT	WT	
solid	m	71	G3	**	**	
acinar	m	65	G3T1bN0V1L1	**	**	
acinar	f	63	G2T1	WT	WT	
papillary	m	55	G2T1mN0	GGT>GTT G12V 12%	WT	
acinar	f	64	G2G3T2N1	WT	WT	
acinar	m	64	G2T1N0	WT	CAA>CAT Q61H 13,5%	
papillary	m	58	G3T2N2	GGT>TGT G12C 24%	WT	
acinar	m	53	G1T1N1	WT	WT	+++/+
acinar	m	55	G2G3T1bN1	GGT>GCT G12A 38%	WT	

acinar	m	66	G2G3T4	GGC>GAC G13D 42,7%	WT	
acinar	f	65	G2T3N0V1L1	GGT>CTT G12L 50%	WT	
acinar	f	60	G2T2m	GGT>GAT G12D 16%	WT	
acinar	m	77	G1T2aN1	GGT>GAT G12D 31%	WT	
acinar	f	48	G1G3T4N3L1	WT	WT	
acinar	f	48	G1G3T4N3	WT	WT	
acinar	m	57	G1T1aN0	GGT>GTT G12V 10,6%	WT	
acinar	m	59	G2N3	WT	WT	
acinar	f	76	G1T3N0L1	GGT>GAT G12D 16,7%	WT	
signet ring	m	62	G2T1aN0	2,1%GGT>AGT G12S	WT	
solid	f	55	G1T1a	WT	CAA>CAC Q61H 35%	
acinar	m	63	G1G3T2aN1	WT	WT	
acinar	m	45	G3T3N0	WT	WT	
acinar	f	70	G1T1a	GGT>GAT G12D 32%	WT	
colloid	m	68	G1T1aN0	GGT>TGT G12C 9%	WT	
papillary	f	80	G3T3N0L1V1	WT	WT	
acinar	f	68	G1T4N0L1	GGT>GTT G12V 9%	WT	
acinar	m	71	G2T1aN0	WT	WT	
papillary	m	84	G3T1bN1	WT	WT	

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