Dissertation

Clinical relevance of immunological biomarkers in NSCLC

submitted by

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Statutory 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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Disclosures

Abstracts from this work were presented at national and international conferences. Parts of the results were published as full paper in April 2024 in Journal of immunotherapy of cancer PMID: 38604811 with the following title:

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1 Abstract

1.1 Background

Recommendation of immune checkpoint inhibitors (ICIs) for the treatment of non-small cell lung cancer (NSCLC) depends on PD-L1 expression on tumor cells. Although PD-L1 expression levels correlate with response to ICI treatment, PD-L1 alone often fails to predict treatment response.

1.2 Methods

In our retrospective cohort of early-stage NSCLC patients, we compared PD-L1 expression in patients with available initial biopsy samples, samples from primary curative surgical and rebiopsy samples in case of subsequent recurrence. In all cases Ventana PD-L1 (SP263) assay was used. We scored PD-L1 expression into clinically relevant groups defined as 0%, 1-49% and ≥50%. Our main interest was the change of PD-L1 score-group between the different matched tumor samples in NSCLC patients.

1.3 Results

In total 395 patients with stage I-III NSCLC were identified forming our main cohort. 136 (34%) out of 395 patients relapsed. For 87 patients of these patients at least two specimens for PD-L1 expression comparison, namely initial biopsy and rebiopsy were available. In 72 cases PD-L1 expression could be compared between all-time points. Between initial preoperative biopsy and matched surgical specimens, a clinically relevant conversion of PD-L1 expression group was found in 25 patients (34.7%). We could not identify a statistically significant influence of neoadjuvant treatment on PD-L1 alteration (p=0.39). When we compared PD-L1 expression from preoperative samples with rebiopsy samples a clinically relevant change in PD-L1 expression was found in 32 (36.8%) out of 87 patients. Similar to the neoadjuvant therapy, adjuvant treatment was also not associated with a change in the PD-L1 expression group (p=0.16). Overall, 39 patients (54.2%) showed at least one change into a different PD-L1 score group. In fourteen patients (19.4%) PD-L1 expression score group changed twice and in five patients (6.9%) PD-L1 score group was different in every analysed sample.

1.4 Conclusion

There is clinically relevant PD-L1 expression change in NSCLC patients during the course of disease. We hypothesize that there is an unmet need for guidelines to define PD-L1 testing strategy including indication for re-assessment, biopsy number and appraisal of surgical specimens.

2 Zusammenfassung:

2.1 Grundlagen und Ziele:

Die Verwendung und Zulassung von Immun-Checkpoint-Inhibitoren (ICIs) zur Behandlung von nicht-kleinzelligem Lungenkrebs (NSCLC) basiert in Ermangelung von Treibermutation auf der PD-L1-Expression im Tumorgewebe. Dennoch versagt die PD-L1 Expression oft dabei, die Ansprechrate auf die Behandlung vorherzusagen.

2.2 Methoden und Ziele:

In dieser Studie haben wir retrospektiv die PD-L1-Expression bei NSCLC Patientinnen und Patienten im frühen Stadium aus der initialen Biopsie mit dem Operationspräperat und im Falle eines Rezidivs aus der Rebiopsie verglichen. Für alle Proben wurde der Ventana PD-L1 (SP263) Immunhistochemie-Assay verwendet. Die PD-L1-Expression wurde basierend auf klinisch relevanten Gruppen (0%, 1-49%, ≥50%) ausgewertet. Der primäre Endpunkt war die Änderung der PD-L1-Score-Gruppe zwischen präoperativen Proben, passenden chirurgischen Präparaten und der Rebiopsie bei Progress.

2.3 Ergebnisse

395 NSCLC Patientinnen und Patienten im Stadium I-III wurden eingeschlossen. In 136 (34%) Patientinnen und Patienten kam es im Verlauf zu einem Rezidiv. Bei 87 Patienten standen mindestens zwei Proben für den Vergleich der PD-L1-Expression zwischen frühem Stadium und der Rebiopsie zur Verfügung. In 72 Fällen war eine longitudinale Analyse zwischen der präoperativen Biopsie, dem chirurgisch resezierten Präparat und der Rebiopsie möglich. Beim Vergleich von präoperativen und passenden chirurgischen Präparaten wurde bei 25 Patienten (34,7%) eine behandlungsrelevante Änderung der PD-L1-Expressionsgruppe festgestellt. Die neoadjuvante Behandlung zeigte keinen signifikanten Einfluss auf die PD-L1-Veränderung (p=0,39). Bei 32 (36,8%) von 87 Fällen wurde eine

Änderung der PD-L1-Gruppe beobachtet, wenn Biopsien des Krankheitsrückfalls mit der initialen Biopsy bei Erstdiagnose verglichen wurden. Die adjuvante Behandlung war ebenfalls nicht mit einer Änderung der PD-L1-Expression assoziiert (p=0,16). 39 Patientinnen und Patienten (54,2%) zeigten im Verlauf der Krankheit mindestens eine Änderung in eine andere PD-L1-Score-Gruppe. Vierzehn Patienten (19,4%) wechselten die PD-L1-Score-Gruppe zweimal, fünf (6,9%) von ihnen befanden sich zu jedem Zeitpunkt in einer anderen Score-Gruppe.

2.4 Schlussfolgerungen

Die PD-L1-Expression zeigt dynamische Veränderungen im Krankheitsverlauf. Eine auf einem Konsens basierende Richtlinie zur Definition einer PD-L1-Teststrategie ist dringend erfordlich. In dieser sollte zukünftig auf die Indikation und das Timing von Rebiopsien, die Anzahl der Probeentnahmen und Schnitte aus chirurgischen Präperaten eingangen werden.

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Abbreviations and Definitions

ALK	Anaplastische Lymphomkinase
BRAF	B-Raf Proto-Oncogene
CEA	Carcinoembryonic Antigen
CONSORT	Consolidated Standards of Reporting Trials
CRC	Colorectal cancer
CK7	Cytokeratine 7
CYFRA-21	Cytokeratin-19-Fragment
ctDNA	Circulating tumor DNA
DFS	Disease free survival
dMMR	Deficient mismatch repair
ECOG-PS	Eastern Cooperative Oncology Group – Performance score
EFS	Event free survival
EGFR	Epidermal Growth Factor Receptor
HER2	Human Epidermal Growth Factor Receptor 2
ICI	Immune checkpoint inhibitors
IHC	immunohistochemistry
KEAP1	Kelch-like ECH-associated protein 1
KRAS	Kirsten Rat Sarcoma Viral Oncogene
LCNEC	Large cell neuroendocrine carcinoma
MET	Mesenchymal-Epithelial Transition Factor

MIS	Microsatellite instability
NSCLC	Non small cell lung cancer
NSE	Neuronspezifische Enolase
NTRK	Neurotrophe Tyrosin-Rezeptor-Kinase
ORR	Objective response rate
OS	Overall survival
PD-1	Programmed death 1
PD-L1	Programmed death ligand 1
PFS	Progression free survival
RET	Rearranged during Transfection
ROS1	c-ros oncogene 1
SCC	Squamous Cell Carcinoma Antigen
SCLC	Small cell lung cancer
STK11	Serin/Threonin-Kinase 11
TPS	Total percentage score
TREG	Regulatory T cells
TTF-1	Thyroid Transcription Factor-1

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3 Introduction

3.1 Epidemiology and Etiology

Lung cancer is the leading cause of cancer-associated mortality and morbidity and a major burden on the health care system in Western nations [1]. Globally an estimated 2.2 million new cases are diagnosed and around 1.8 million deaths were attributed to lung cancer in 2020 alone. In Austria incidence of lung cancer is still rising. In 2022 5200 patients were diagnosed with lung cancer, which corresponds to an increase of around 600 cases compared to the previous survey by statistic Austria. This increase is caused by rising lung cancer diagnoses in women, while incidence in men is falling slowly.[2] The main risk factor for the development of lung cancer is active and passive smoking. Tabacco smoke contains over 4,000 chemicals, including 69 established carcinogens and other toxicants associated with lung cancer.[3,4] Unsurprisingly smoking is associated with a 20-fold increased risk for lung cancer development and 15% of smokers will develop lung cancer during their lifetime. It is estimated that up to 85% to 90% of lung cancer cases are associated with smoking. The risk of lung cancer development in smokers increases with the total amount of smoking measured in pack years. Interestingly, the rate of smoking associated lung cancer is higher in male (91%) and lower in female (65%) patients, which is partly explained by the higher number of driver mutations in female lung cancer patients.[5] However, due to the increasing number of smoking women this gap is closing. [1,2] Apart from tabacco consumption lung cancer may be caused by exposure to other carcinogens or toxins. An estimated 9 to 15% of lung cancer cases are attributed to occupational exposure including exposure to asbestos, silicate, chrom, nickel, beryllium, ionizing radiation and arsenic. [6] Other risk factor for lung cancer development include air pollution and nutrition. [7]

3.2 Histology and Classification

Lung cancer can be divided into two subtypes based on its histology. The main histological subtypes in lung cancer are non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). This main differentiation is essential for it guides treatment and predicts outcome.

3.2.1.1 NSCLC

NSCLC accounts for up to 85% of lung cancer [8]. The NSCLC can be divided in several subtypes. The most frequent subtype is the adenocarcinoma (45%), and squamous cell carcinoma (25%).[2] However, the 2021 WHO classification of lung tumors also includes rare lung tumors including large cell carcinoma, adenosquamous carcinoma and sarcomatoid carcinoma [9]. Previous results suggested that NSCLC cells originate in basal like stem cells. Recent studies, however, have shown that alveolar type 2 and club cells can also transform into NSCLC. To this day the cell origin of NSCLC remains elusive.

3.2.1.2 Adenocarcinoma of the lung

The adenocarcinoma is the most prevalent subtype of NSCLC and comprises up to 40% of lung cancer cases. [2,10]. Adenocarcinoma in contrast to squamous cell carcinoma is more likely to originate in the peripheral bronchial tree and may remain asymptomatic for a longer time period. Adenocarcinomas are more frequent in non or occasional smoker in part due to the higher number of driver mutations when compared to other histological subtypes.

The WHO classification of lung tumors defines three stages of development in adenocarcinoma of the lung based on growth pattern and invasion.[9] The earliest form is the so called adenocarcinoma in situ (AIS). It is a precursor of the invasive adenocarcinoma and is defined as slow growing carcinoma with a maximal diameter of 3cm and missing of interstitial or vascular invasion. If this tumor starts to invade interstitial structures up to 5 mm but remains localized it is called minimal invasive adenocarcinoma (MIA). This tumor usually grows lepidic, following the lining of the alveolar structures. If vascular or lymphatic vessels or pleural surfaces are invaded minimal invasive adenocarcinoma has evolved to invasive adenocarcinoma. The invasive adenocarcinoma is characterised by infiltration of surrounding tissue of more than 5 mm, invasion of vascular or pleural spaces and or diameter of more than 3 cm. Histologically there are several subtypes of invasive adenocarcinoma. The most prevalent are mucinous and non mucinous invasive adenocarcinoma. However, there are other rare variants that show colloid, fetal, and enteric differentiation.

Grading of adenocarcinoma of the lung is currently performed by analysis of the single most prevalent histological pattern. Lepidic adenocarcinomas are usually low grade (G1). Lepidic adenocarcinoma follow the lining of the alveolar sack and have a better prognosis. Acinar and papillary adenocarcinoma are graded intermediate (G2), while solid or micropapillary tumors are high grad (G3) and are associated with the worst prognosis.[11,12] Typical immunohistochemical markers for adenocarcinoma of the lung are Thyroid Transcription Factor-1 (TTF-1), Napsin A and Cytokeratin 7 (CK7).

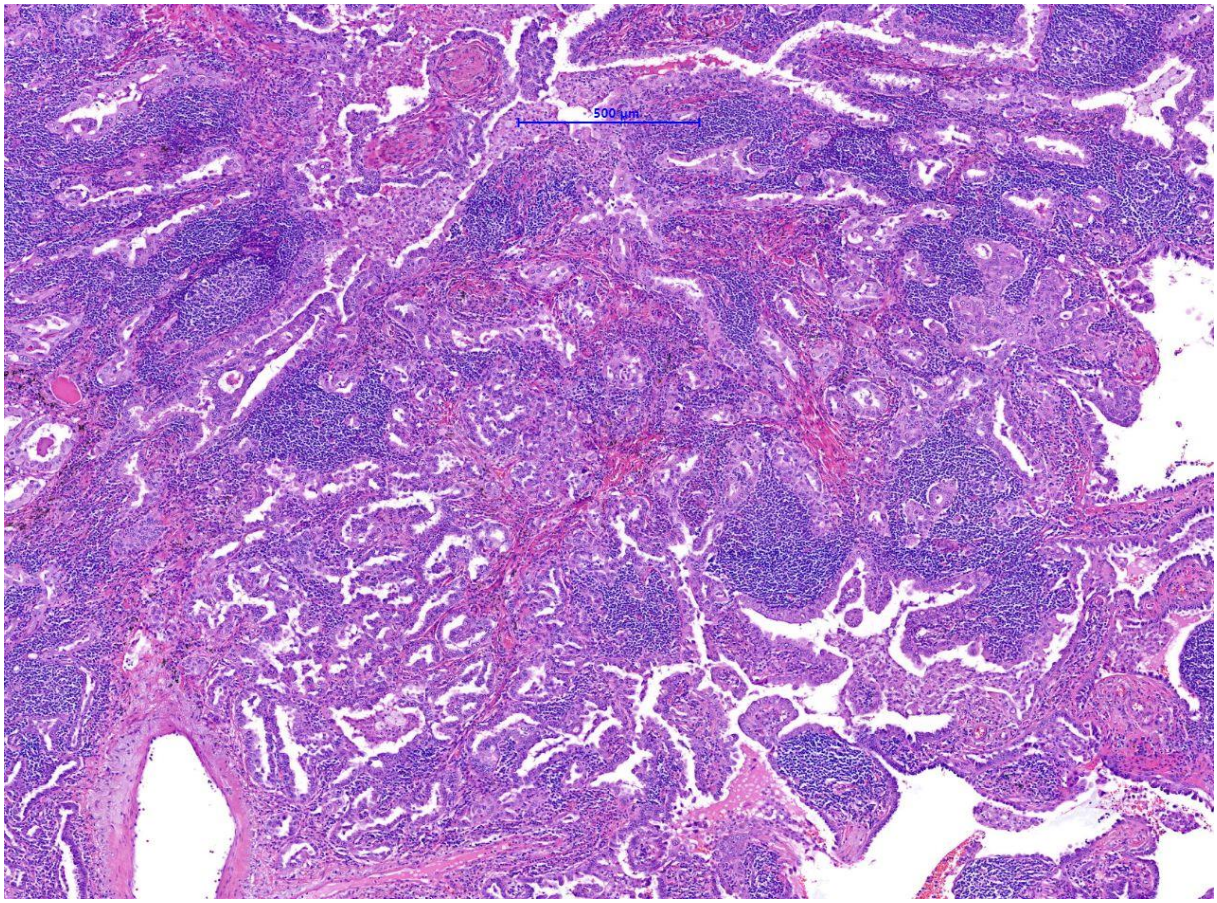

Figure 1 *Adenocarcinoma of acinary type with pronounced lymphatic follicules. This slide was generously made available with the kind support of Prof. Dr. Luka Brcic. Head of pulmonary pathology Medical University Graz.*

3.2.1.3 Squamous cell carcinoma

Squamous cell carcinoma account for 20-30% of NSCLC.[10] It is associated with heavy smoking and typically originates in the epithelium of central airways.[13] Therefore, clinical signs are seen earlier than in peripheral NSCLC. Still most patients are diagnosed in a progressed stage and prognosis is even worse than adenocarcinoma due to fewer treatment options. Main reason is the lack of targetable

mutations when compared to adenocarcinoma of the lung. Like adenocarcinoma of the lung a sequence of neoplastic alterations is described. In the first step squamous metaplasia is formed, which may evolve into squamous dysplasia and finally into carcinoma in situ. In cases of invasive growth this carcinoma in situ has evolved to invasive squamous cell carcinoma. Histologically based on differentiation of squamous cell carcinoma keratinizing, non-keratinizing and basaloid forms are described. Squamous cell carcinoma is defined as keratinizing if there is any sign of keratinizing and basaloid if there are more than 50% of basaloid cells within the tumor. While there is no convincing evidence that keratination has an impact on prognosis some studies found that basaloid differentiation is associated with poor prognosis.[14,15] This finding however is disputed by other publications that found no impact on prognosis.[16] Immunohistochemical markers for squamous cell carcinoma of the lung are p40 and CK5/6 the transcription factors SRY-box 2 (SOX2) and p63.[13]

3.2.1.4 Rare subtypes of NSCLC

Large cell carcinoma

In the 2004 WHO classification, large cell carcinomas were a heterogeneous group of tumors that included LCNEC, basaloid carcinoma, lymphoepithelioma-like carcinoma, clear cell carcinoma, and large cell carcinoma with rhabdoid phenotype. However, recent molecular genetic analysis found that most of these subtypes showed either adeno- or squamous cell genotype.[17] These findings and introduction of TTF-1 lead to reclassification of most large cell carcinoma.[18] Today large cell carcinoma is a rare phenotype.

Sacromatoid carcinoma

The term sarcomatoid carcinoma is an umbrella term and consists of pleomorphic carcinoma, carcinosarcoma, and pulmonary blastoma. All forms are rare and only account for less than 1% of lung cancer cases. [19] They are associated with a poor prognosis and there is no specific treatment option in progressive disease.[20]

Pleomorphic Carcinoma is defined as a low differentiated non squamous cell carcinoma of the lung with at least 10% spindle and or giant cells. As KRAS and EGFR mutation may occur in these tumors next genome sequencing (NGS) is recommended.[9] Carcinosarcoma consists of a combination of NSCLC and typical sarcoma histological patterns including rhabdomyosarcoma, chrondrosarcoma and osteosarcoma. Pulmonary blastoma is very rare (0,5% of NSCLC) biphasic tumor that consists of a combination of epithelial and a mesenchymal malignant component.[21] The prognosis of this tumor is

especially poor. Furthermore, due to the rarity of this tumor there is no standard treatment recommendation in the advanced state. Treatment usually consists of platinumbased chemotherapy and radiotherapy if curative treatment is not possible. There is no evidence for checkpoint inhibition in pulmonary blastoma, however some case series found promising results.[22]

3.2.2 Neuroendocrine carcinomas of the lung

Overall neuroendocrine neoplasms account for around 20% of lung tumors. The most frequent neuroendocrine neoplasm is SCLC followed by carcinoids, and large cell neuroendocrine carinoma (LCNEC), that account for around 15%, 2% and 3% respectively.[23] The defining WHO criteria for differentiation of these neuroendocrine neoplasms is based on the distinct morphology and proliferation rates. Typical neuroendocrine morphology consists of organoid or trabecular patterns, rosette-like structures, or peripheral palisading.[23,24] In terms of proliferation rates low mitotic count (below 2 mitosis per 2 mm² often defined as one high powered filed (HPF)) is a very suggestive of carcinoids. If necrosis is present but mitotic rate is below 10 per HPF atypical carcinoid is the most likely underlying diagnosis. A mitosis count higher than 10 mitosis per 2 mm² is considered high grade which is highly suggestive of SCLC or LCNEC. [25] Typically used immunohistochemical markers in neuroendocrine tumors are neural cell adhesion molecule (NCAM), chromogranin and synaptophysin.

3.2.2.1 SCLC

Small cell lung cancer (SCLC) is a poorly differentiated, very aggressive neuroendocrine carcinoma of the lung. It accounts for up to 15% to 20% of patients with lung cancer and is associated with heavy smoking.[26] Historically SCLC was first described in 1879 by Harting and Hesse. They described SCLC as a form of lymphosarcoma. [27] In the early 20th century it was rebranded as “oat-cell carcinoma” due to its densely grouped cells that reminds of oat.[28] Progress in molecular pathology lead to its inclusion in the category of neuroendocrine carcinoma of the lung in the WHO classification of tumors of the lung.[9] SCLC originates from pulmonary neuroendocrine cells (PNECs). [29] However, when compared to other forms of lung cancer there is no definitive proof of a precancerous lesion in SCLC. Preclinical models suggest that SCLC develops directly out of molecular damaged but still normal or mildly abnormal neuroendocrine cells in lung tissue without complex histologic sequence.

In almost all patients SCLC is diagnosed in a metastasised stage due to the extremely rapid progression. Prognosis is poor even in very early cases (VLD). Even in this early stage of disease surgery is only recommended in selected cases and adjuvant chemotherapy is recommended in all cases.[30] Patients in localised stage of disease (LD) are treated with chemotherapy and radiation. 5-year overall survival in this group may reach up to 20%.[31] Although this tumor usually responds well to chemotherapy, early relaps with resistant disease leads to an especially poor prognosis with an estimated 5 year overall survival of around 5 to 10 %.[32] Although checkpoint inhibitors are approved for SCLC, benefit for patients is mediocre. Still both the Impower 133 and the CASPIAN trial found increased overall survival (13 vs 10,3 months) and (12,3 vs 10,3 months) if patients received checkpoint inhibitors in combination with platinum-based chemotherapy.[33,34] However, a minority of patients seems to benefit even after years of treatment.[35,36] Still impact of check point inhibition in SCLC is limited. Although new promising approaches like DLL3-antibodies are currently investigated in clinical trials, no therapy has yet had a significant impact on the overall survival of patients with SCLC.[37]

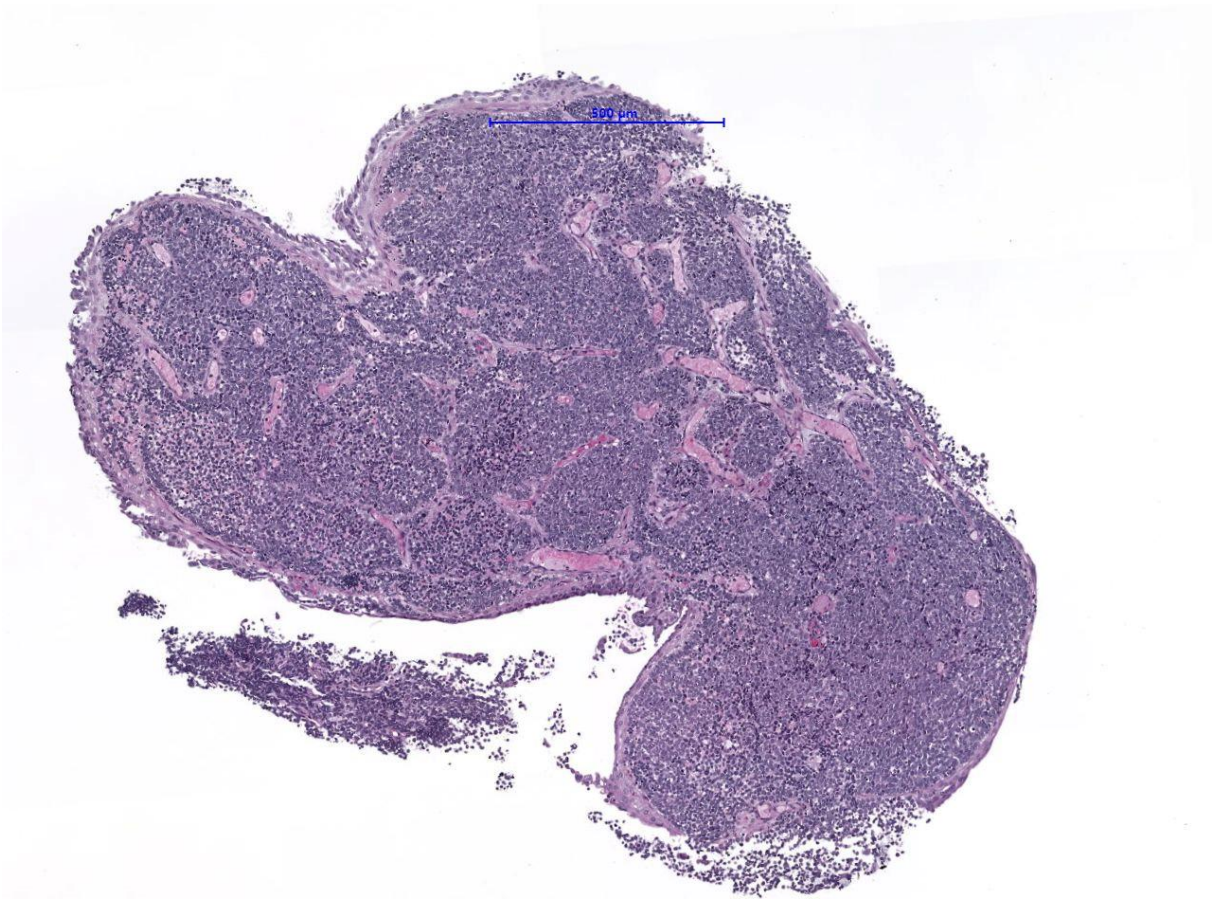

Figure 2 *SCLC - This slide was generously made available with the kind support of Prof. Dr. Luka Brcic. Head of pulmonary pathology Medical University Graz.*

3.2.3 Large cell neuroendocrine carcinoma

Large cell neuroendocrine carcinoma of the lung (LCNEC) is a rare and highly aggressive form of lung cancer. It shares similarities with SCLC and NSCLC. The prognosis of LCNEC is comparable to SCLC with a median overall survival of around 9 months.

Histopathological LCNEC shows a high grad neuroendocrine morphology with a large cell size, prominent nucleoli and abundant cytoplasm in addition to a high mitosis count of more than 10 per HPF and necrosis.[38] In addition to the typical morphology at least one neuroendocrine marker have to be positive. However, diagnosis may be very difficult in small biopsies due to crushing artifacts.[38]

Rekhtman and colleagues found that within the entity of LCNEC there are two distinctive subtypes. A SCLC like subtype and a NSCLC like subtype that accounted for 40% and around 60% respectively. While the SCLC subtype showed higher rates of TP53 and RB1 alterations, the NSCLC like subtype was lacking RB1 and TP53 and showed NSCLC like alterations like STK11, KRAS and KEAP-1. [39] This finding may have clinical significance, as registry data suggest that LCNEC with SCLC like histology showed higher ORR when treated with platin-etoposide chemotherapy, while NSCLC like LCNEC showed higher ORR when treated with platin-gemcitabine or taxane chemotherapy.[40]

Due to the rarity of the disease, there is no approved treatment regimen in LCNEC. However, at least in localised disease there is sufficient to recommend surgery. Compared to chemotherapy and radiotherapy alone, patients who undergo surgery in stage I to III show an strongly improved 5 year median overall survival (18–75%).[41,42]. This high variability in prognosis is very likely caused by the high heterogeneity of LCNEC.

Post surgery, several studies found that adjuvant chemotherapy was associated with statistically significant improvement in prognosis compared to surgery alone.[43] The most often used adjuvant chemotherapy regiment was cisplatin in combination with etoposide[44].

In advanced stage contrary to previous recommendations next genome sequencing should be performed since some LCNEC especially with adenocarcinoma differentiation may harbor targetable mutations (especially EGFR).[45] Checkpoint inhibitors showed promising results in several case series. However, ICI is still not approved in treatment of LCNEC. [46]

3.2.4 Carcinoids

Carcinoids of the lung are slow growing semi malign neuroendocrine tumors. They account for up to 2% of neuroendocrine tumors. There are two main types of carcinoids of the lung, which are the typical and atypical carcinoid. The typical carcinoid is the more common and less aggressive form. The ratio

between typical and atypical carcinoid is around 10:1.[9] Histologically, lung carcinoids exhibit uniform, round cells with moderate cytoplasm, arranged in nests or trabeculae. Mitotic activity is generally low in typical carcinoids but higher in atypical ones, and necrosis may be present in the latter. A mitotic rate of more than 2 per mm^2 or the presence of necrosis are diagnostic criteria of the atypical carcinoid. The overall prognosis is far better than other forms of neuroendocrine tumors of the lung. However, the likelihood of metastasis depends on the histological type. While the typical carcinoid has a low risk of metastasizing of 15%, the atypical carcinoid has a higher risk of up to 50%. Therefore, surgery is usually recommended in all patients with localised disease.[47]

3.3 Diagnosis of lung cancer

All patients in which lung cancer is suspected should undergo specific anamnesis, clinical examination and laboratory testing and imaging and if in case of high probability of lung cancer invasive diagnostic procedures like bronchoscopy.

3.3.1 Anamnesis

Anamnesis should consist of previous diseases and medication, family history, occupational exposure and especially smoking history. Smokers should be asked about daily consumption to quantify tobacco exposure in pack years. Furthermore, suggestive symptoms of lung cancer like newly onset or worsening cough, hemoptysis, weight-loss, weakness, fever and pain. Finally, physical capability should be assessed in all patients using ECOG or Karnofsky Index (see table 1).

Karnofsky Status	Karnofsky Grade	ECOG Grade	ECOG Status
Normal. Patients show no signs or symptoms of disease	100	0	Patients are active and able to carry out all normal performance in daily life without restriction
Minor signs or symptoms of disease, but patients are not restricted in daily life	90	0	
Normal activity is possible but requires effort	80	1	Patients are restricted in physically demanding daily activity but ambulatory and able to carry out work of non-demanding nature, e.g., light house work or office work
Patients are able to care for themselves, but are unable to carry on normal activity or to do active work	70	1	
Patients require occasional assistance, but are able to care for most of his needs	60	2	Patients are unable to carry out any work activities. However, they remain ambulatory and are capable of selfcare. They are up and about more than 50%

			of waking hours but cannot carry out any work activities.
Patients requires considerable assistance and frequent medical care	50	2	
Patients are disabled and requires care to manage their daily life	40	3	Patients are capable of only limited selfcare and are confined to bed or chair more than 50% of waking hours
Patients are severly disabled. Hospitalisation is indicated though death is not imminent	30	3	
Patients are very sick and Hospitalisation and active supportive treatment is necessary.	20	4	Patients are very sick and Hospitalisation is indicated.
Patients are moribund	10	4	
Dead	0	5	Dead

Table 1 *Karnofski and ECOG-Performance Score adapted from Oken et. al. "Toxicity and response criteria of the eastern cooperative oncology group" Am J Clin Oncol 5:649-655, 1982*

3.3.2 Clinical presentation

In the early stages of the disease patients are frequently asymptomatic. Although most patients are diagnosed in advanced stages, at diagnosis up to 70% of patients present with mild symptoms [48]. Most relevant clinical signs and their clinical frequencies are shown in table 2. One third of symptoms are usually associated with the primary tumor, one third is caused by systemic impact of lung cancer, while another third of clinical signs can be attributed to distant metastasis [49].

Symptoms	Frequency
Cough	8-75%
Weight-loss	0-68%
Breathlessness	3-60%
Chest pain	20-49%
Hempotysis	6-35%
Pain	6-25%
Clubbing	0-20%
Fever	0-20%
Weakness	0-10%

Table 2 *Symptoms in lung cancer patients adapted from "Spiro et al. Initial evaluation of the patient with lung cancer: symptoms, signs, laboratory tests, and paraneoplastic syndromes: ACCP evidenced-based clinical practice guidelines (2nd edition)"*[49]

3.3.3 Clinical signs of local tumor growth

Most prevalent clinical sign associated with local tumor growth is newly onset or worsened cough. Up to 75% of patients do suffer from cough at diagnosis. Dyspnoea, thoracic pain and hemoptysis are frequently present. Hemoptysis is present in up to 35% of cases and often caused by tumors growing in the central airways. Therefore this clinical sign must prompt further investigation, especially in middle-aged patients with smoking history even in cases of unremarkable chest x ray.[50] Rare clinical signs include dysphagia, stridor or superior vena cava syndrome caused by tumor compression.

3.3.4 Clinical signs of intrathoracic tumor growth

Local tumor progression may lead to infiltration of thoracic organs including the mediastinum, the pleura and the chest wall and nerve structures. Clinical signs include hoarseness, caused by compression of the nervus recurrens, horner syndrome caused by infiltration of the plexus brachialis in Pancoast tumors and superior vena-cava syndrome caused by compression of the vena cava superior.

Furthermore, thoracal pain is a common clinical condition that is caused by invasion of the pleura and chest wall. Up to 50% of patients develop this complication in the course of their illness.

3.3.5 Clinical signs of distant metastasis and systemic disease

One third of clinical signs is caused by distant metastasis and systemic disease. These include pain caused by metastasis especially in bone metastasis, headache, disorientation, seizure and focal neurological deficit, caused by brain metastasis, icterus and liver failure caused by liver metastasis as well as asthenia and weight loss.[49] Asthenia and weight loss are very often associated with distant metastasis and have to be inquired about in all patients.[51]

3.3.6 Paraneoplastic syndromes

Paraneoplastic syndromes are defined as specific clinical signs that are associated with malignant disease however without direct connection to tumor growth. These syndromes are caused by cytokines or hormones released by tumor or the immune system as response to tumor growth and affect up to 10% of patients.

Paraneoplastic syndromes may be grouped in several categories. These include hematologic, endocrine, neurological, dermatological, skeletal, renal, metabolic and vasculitis like syndromes. The most relevant paraneoplastic syndromes are summarized in table 3.

Hematologic Syndromes	Chronic anemia Leukocytosis with eosinophilia Leukemoid reaction Autoimmune hemolytic anemia Erythrocytosis Thrombocytosis, -penia Microangiopathic hemolytic anemia Coagulopathy Thrombophlebitis
Endocrine Syndromes	Syndrome of inappropriate ADH secretion (SIADH) Cushing's syndrome Hypercalcemia Hypercalcitoninemia Gynecomastia Hypoglycemia Hyperthyroidism
Neurological syndromes	Subacute sensory neuropathy Mononeuritis multiplex Intestinal pseudo-obstruction Lambert-Eaton syndrome Myasthenia gravis Encephalomyelitis Necrotizing myelopathy Tumor-associated retinopathy Guillain-Barré syndrome Limbic encephalopathy Opsoclonus-myoclonus Subacute myelopathy

Dermatologic Syndromes	Erythema gyratum repens Erythema multiforme Tylosis (Howel-Evans-Syndrom) Erythroderma Sweet's syndrome Acanthosis nigricans Necrolytic migratory erythema Exfoliative dermatitis Pruritus and urticaria
Skeletal Syndromes	Hypertrophic osteoarthropathy (Pierre-Marie-Bamberger syndrome) Clubbing
Renal Syndromes	Glomerulonephritis Nephrotic syndrome
Collagenous or vasculitis like syndromes	Dermatomyositis Polymyositis Vasculitis Systemic lupus erythematosus (SLE)
Metabolic Syndromes	Lactic acidosis Hypouricemia

Table 3 *Paraneoplastic syndromes in lung cancer adapted from Kreuter M. et. al "Diagnostik des Bronchialkarzinoms" Pneumologe 2008*[52]

3.3.7 Tumormarkers

Several publications found that tumor markers like Squamous Cell Carcinoma Antigen (SCC), Carcinoembryonic Antigen (CEA), Neurospecific Enolase (NSE) and Cytokeratin-19-Fragment (CYFRA 21-1) reach low sensitivity of around 20 to 70% percent for detection of lung cancer. Due to

the low positive predictive value (around 20%) and consequently high rate of false positive results tumor markers can still not be recommended in screening populations.[53] Additionally in cases of clinical suspicion tumor markers are not reliable enough to rule out or confirm diagnosis. Therefore, tumor markers are not recommended for clinical routine in diagnosis of NSCLC or SCLC. However, these tumor markers may be used for treatment response and surveillance. New biomarkers like circulating tumor DNA (ctDNA) are evaluated in several clinical trials. However to date ctDNA panels are not standardized and not recommended for routine clinical use.

3.3.8 Imaging in lung cancer

3.3.8.1 Computed tomography

Contrast enhanced computed tomography is the most important imaging modality in the diagnosis and staging of lung cancer and guides essential diagnostic interventions like bronchoscopy or transthoracic biopsies. To exclude neoplastic infiltration of organs slice thickness should not exceed 2 mm. For staging it is recommended to include the upper abdomen to include liver and adrenal glandes, for these organs are typical location for metastasis. Evaluation of the liver requires scans in the portal venous phase which is usually reached 70 seconds after administration of contrast. Although some radiological signs may increase probability of malignancy there is no specific radio-morphologic pattern for lung cancer. Possible radiologic patterns include ground glas opacities, solid and subsolid pulmonary nodules, central lesions with atelectasis and consolidation.

3.3.8.2 Lung cancer screening

One important reason for the high mortality of lung cancer is the fact that diagnosis is usually performed in later stages of the disease. Therefore, establishing lung cancer screening programs was an early goal for control of lung cancer. However, prior screening trials based on annual chest x-ray and sputum cytology failed to show statistically significant reduction in lung cancer mortality. However, the development of low dose spiral CT scans led to invigorated interest in screening programs. To date the results of eight randomized low dose CT scan-based screening trials were published. Most European trials however, were underpowered for analysis of mortality. The NELSON study in the Netherlands and the NLST study in the USA are the largest cohort studies to date. Both found statistically significant reduction in mortality in a high-risk cohort. The NLST study defined the study cohort as asymptomatic active or ex-smokers that are at least 55 to 75 years old that accumulated at least 30 pack years. In total 53,454 participants were enrolled in the study. The participants of the intervention group received a low dose CT scan once per year over a 3-year period. In the control group participants received chest x-ray.

The study found a reduction in lung cancer mortality of 20% (95% confidence interval [CI], 6.8–26.7; p = 0.004) after 6.5-years follow-up.[54] The NELSON study reported a rate ratio for lung cancer associated mortality of 0.76 (95% CI, 0.61–0.94; p = 0.01) in the screening cohort. Interestingly, this reduction in mortality was even more pronounced in the subgroup of female participants.[55] Still screening CT scans always lead to a relevant number of false positive cases. In the NELSON study around 23% of suspect nodules were false positive causing unnecessary cost and potential harm for patients. Furthermore, screening programs cause a significant economic burden on already strained health budgets. Still leading scientific societies recommend the implementation of low dose screening CT scans in high-risk populations.

3.3.8.3 PET-CT

Fluorodeoxyglucose positron emission tomography computed tomography (PET-CT) is a non-invasive imaging technic for the accurate staging of lung cancer. The most common tracer is Fluoro-2-Desoxy-D-Glukose (F18-FDG). As glucose is in high demand in tissues with high metabolic rate, this tracer marked glucose molecule accumulates as well. Positron decay of F18FDG can then be measured as standardized uptake volume (SUV). High concentrations of F-18-FDG is physiological in some organs like the brain or hearth muscle. However, elevated uptake outside of these organs is highly suggestive of malignancy, while low uptake may be present in areas of inflammation or benign diseases like sarcoidosis. Depending on the clinical context F18-FDG PET-CT scans reach higher sensitivity and specificity for malignancy than contrast CT scans. However, the main use of FDG PET-CT is not in diagnosis but in staging of lung cancer. [56] A meta-analysis by Schmidt and colleagues found pooled sensitivity for malignancy in mediastinal lymph nodes ranging from 77% to 90% [57]. The low SUV cut-off of 2.5, however, lead to a relatively low specificity of around 79%. Still the high sensitivity led to a high NPV of around 90%. Which is in line with previous meta-analysis.[58] Wang and colleagues found that in early stage lung cancer patients with PET-negative nodal status NPV reached 94% in patients with tumors of less than 3cm in size [58]. However, diagnostic accuracy for nodal status may be impaired in some situations. For example, larger primary and central tumors are associated with higher rates of occult N2. Especially central tumors are associated with far higher rate of PET-negative occult N2 disease (2.9% vs. 21.6%), which lead to a recommendation for invasive testing in the ESTS guidelines[59]. On the other hand, a meta-analysis by De-Langen et al found that mediastinal lymph nodes of less than 10mm size and negative FDG PET-CT scan were malignant in only 5% cases.[60] This finding suggests that size and PET-scan may exclude malignancy reliable enough to avoid invasive testing. However, in lymph nodes of 15mm and negative FDG PET-CT scan up to 21% showed malignant infiltration that was missed by PET-scan. After surgery several authors identified no additional benefit for PET-CT in follow up care of lung cancer patients.[61] The most important benefit of PET-CT is the detection of occult extra-thoracic metastases. In comparison to previous staging imaging PET-CT detects occult metastases in up to 15% of patients.[62] Especially sensitivity for metastases in bone, liver and adrenal glandes are far superior than other forms of imaging. In case of bone metastases, a meta-analysis of 17 studies demonstrated a pooled sensitivity and specificity of 92% and 98%, respectively, for PET-CT, which by far exceeds results of bone scintigraphy. The same was true for MRT, which only reached a pooled sensitivity of 77%. [63] In follow up care after resection of lung cancer, PET-CT did not show higher sensitivity than contrast enhanced CT scans for recurrence of disease. PET-CT should therefore be no part of clinical routine in follow up care.[64]

3.3.8.4 Bone Scintigraphy

Bone scinitgraphy is a widespread diagnostic tool to bone metastasis in lung cancer. However, a recent meta-analysis of six prospective trials found that bone scan results were false positive in up to 38% leading to a positive predictive value of only 32%. The high sensitivity of bone scans of up to 82% lead to an acceptable negative predictive value of 90%. The authors concluded that in patients with negative scan results bone metastasis may be excluded with sufficient probability. In contrast, in patients with positive results further diagnostic tests are necessary. [51]

3.3.9 Bronchoscopy

Bronchoscopy is an important tool for diagnosis and staging of lung cancer. Depending on the localisation it has an excellent diagnostic yield. In the past this was only true for central lung tumors [65], while diagnostic results for peripheral nodules in the lung was far lower. [66] This holds still true if lung nodules are biopsied by transbronchial biopsy guided only by fluoroscopy. [66] Diagnostic yield was slightly increased by using multiple endoscopic biopsy techniques including brush, needle, and forceps biopsy are combined.[67] Still, the main issue of proper positioning of the bronchoscope remained unsolved. A study by Modoni et al. found that in fluoroscopy guided bronchoscopy the peripheral lesion led to diagnosis in only 53% of cases. Diagnostic yield was higher if lesions were larger or showed endobronchial connection, but results remained unsatisfactory.[68]

The development of new navigation bronchoscopy techniques, lead to remarkable improvement in diagnostic accuracy of peripheral lung lesions. Wang-Memoli et al. found that electromagnetic navigation bronchoscopy was able to obtain navigation success in 95% and a diagnostic yield of 70%.[69] Other navigation techniques like cone beam ct navigation bronchoscopy and robotic bronchoscopy reached even higher diagnostic yield but are associated with higher economic and technical demands.[70]

Furthermore, hilar and mediastinal lymph nodes may be reached by EBUS-TBNA which may provide diagnosis even in cases where the main peripheral lesion was not reached and provides important information of local progression of the tumor. A recent meta-analysis found that in cases of enlarged or FDG-PET-CT positive mediastinal lymph nodes EBUS-TBNA lead to cytologic diagnosis in up to 90% of cases.[71]

Appart from diagnosis of lung cancer EBUS-TBNA plays an integral part in staging of mediastinal lymph nodes in lung cancer. Several studies found that EBUS-TBNA has high sensitivity and NPV for staging of mediastinal lymph nodes of up to 89% and 91% respectively [68,72] Furthermore, when

compared to mediastinoscopy EBUS-TBNA is equivalent in diagnostic yield and associated with less interventional risk [73].

3.4 Lung cancer staging

Staging of lung cancer is an essential part of initial evaluation of all lung cancer patients. Accurate staging of patients allows for estimation of prognosis and planning of treatment. Staging of lung cancer should be based on the 8th edition of UICC/AJCC TNM-system in all patients.[74] However, in SCLC due to its ease of use the staging system of the Veterans Administration Lung Study is often used in clinical routine.[75] If TNM 8th edition is used for SCLC one main difference is the fact that differentiation of stage IV (IVA,B) is not necessary, since there is no prognostic value in curative treatment in oligometastatic stage of SCLC. [76]

T classification	Tumor size
Tis (AIS)	Pure GGN ≤ 3 cm
T1mi	≤ 0.5 cm solid part within part-solid tumor total size ≤ 3 cm
T1a	Pure GGN > 3 cm or
	≤ 1 cm solid tumor or subsolid tumor with solid component ≤ 1 cm
T1b	1.1–2.0 cm solid tumor or subsolid tumor with solid part of 1.1–2.0 cm
T1c	2.1–3.0 cm solid tumor or subsolid tumor with solid part of 2.1–3.0 cm
T2a	Tumor size of 3-4 cm or
	Tumor involves main bronchus
T2b	Tumor size of 4-5 cm or
	Total/partial atelectasis or
	Involves visceral pleura
T3	Tumor size of 5-7 cm or
	Separate tumor nodules in the same lobe as the primary or
	Involves parietal pleura or
	Involves parietal pericardium
	Involves Chest wall
	Involves Phrenic nerve
T4	Tumor size of > 7 cm
	Involves diaphragm
	Involves mediastinal structures (trachea, great vessels, heart, recurrent laryngeal nerve, esophagus) or

	Involves Carina or
	Involves Vertebral body or
	Involves visceral pericardium

Table 4 *Tumor size and spread (T-classification) adapted from "tnm classification of malignant tumors 8th edition" James D. Brierley et. al.* [77].*Ground glass nodule (GGN) is defined as sub solid nodule.*

N classification	Lymph node invasion
N0	No lymph node metastases
N1	Ipsilateral peripheral, intrapulmonary or hilar node metastases
N2	Ipsilateral mediastinal or subcarinal lymph node metastases
N3	Ipsilateral or contralateral supraclavicular or contralateral hilar or mediastinal, lymph nodes metastases

Table 5 *Lymph node invasion (N-classification) adapted from "tnm classification of malignant tumors 8th edition" James D. Brierley et. al.* [77]

M classification	Documented metastases
M0	No distal metastases
M1a	Malignant pleural or pericardial effusion Intrathoracic metastases Pleural metastases
M1b	Single extrathoracic metastasis in a single organ
M1c	Multiple extrathoracic metastasis

Table 6 *Documented metastases (M-classification) adapted from "tnm classification of malignant tumors 8th edition" James D. Brierley et. al. [68]*

	N0	N1	N2	N3
Tis	0			
T1mi	IA1			
T1a	IA1	IIB	IIIA	IIIB
T1b	IA2	IIB	IIIA	IIIB
T1c	IA3	IIB	IIIA	IIIB
T2a	IB	IIB	IIIA	IIIB
T2b	IIA	IIB	IIIA	IIIB
T3	IIB	IIIA	IIIB	IIIC
T4	IIIA	IIIA	IIIB	IIIC
M1a		IV A		
M1b		IV A		
M1c		IV B		

Table 7 *Tumor Stage (TNM-classification) adapted from "tnm classification of malignant tumors 8th edition" James D. Brierley et. al. [68]*

3.5 Therapy of lung cancer

Treatment of lung cancer depends on the histology and stage. The therapeutic options for SCLC deviate from treatment of NSCLC and will not be further elaborated. Localised disease of NSCLC may be treated curatively by surgery or radiotherapy, while metastatic disease is currently treated with systemic agents in most cases. Some patients in oligometastatic stage may benefit from curative treatment. However, study results in this stage are contradictory, which will be discussed below.

3.5.1 NSCLC localised disease

In stages IA and IB surgery is the treatment option of choice and leads to remarkable 5 year overall survival of 80-93% and 71% respectively. Adjuvant treatment may be considered for stage IB with additional risk factors like solid or micropapillary growth or L1 status. However, this recommendation is not based on prospective data. In stage II surgery is gold standard of treatment as well and leads to 5 year overall survival of 50-60%. Adjuvant treatment is recommended and is associated with improvement in survival of up to 11.6% after 5 years. Postoperative radiotherapy (PORT) is only recommended in R1 when surgery may not be repeated or mediastinal lymph node capsule breach. Based on the ALINA and ADAURA study in patients with ALK or EGFR mutation adjuvant treatment with TKI are recommended. In both cases TKI-treatment leads to strong improvement in event free survival and overall survival.[78,79] Neoadjuvant treatment may be indicated in some patients with stage II disease. Several studies including Keynote 671, AEGEAN, checkmate 816, NADIM and Checkmate 77T found significant improvement in event free survival even in Stage II disease. In all these studies patients received a combination of chemotherapy and PD or PD-L1 targeting immune checkpoint inhibitor (ICI). In stage III risk of recurrence after surgery increases and surgery is not the best treatment option for all patients. Patients with stage IIIA benefit from surgery but based on studies like checkmate 816 neoadjuvant treatment should be recommended in all patients.[80] Surgery in this group of patients leads to 5-year overall survival between 15 and 40%. To date adjuvant therapy is recommended in all patients that did not receive neoadjuvant treatment. Adjuvant treatment should consist of up to 4 cycles of cisplatin and one additional agent. After completion of adjuvant chemotherapy patients should receive adjuvant ICI treatment if they did not receive neoadjuvant immunochemotherapy based on the impower 010 study or PEARLS study. [81,82] Patients with ALK or EGFR mutations were excluded from neoadjuvant immunochemotherapy studies. If these patients would benefit from this treatment is unclear however previous studies found no added benefit of immunotherapy. If stage IIIA patients with EGFR or ALK mutations undergo surgery, they should receive adjuvant TKI treatment as mentioned above.

In stage IIIB and IIIC surgery is not associated with increased survival and should therefore only be performed after case discussion in interdisciplinary tumor boards. However, it must be pointed out that

neoadjuvant studies like AEGEAN, checkmate 77T and NADIM included a minority of stage IIIB patients with promising results. Therefore, progression in neoadjuvant treatment may lead to a higher rate of surgery in localised progressed stage III patients in the future. Previously stage IIIB and IIIC patients received concomitant radio chemotherapy with consolidation immunotherapy in positive PD-L1 expression. This led to 3 year overall survival of 63% [83] Patients with EGFR and ALK mutations should be evaluated for consolidation TKI treatment after radiochemotherapy. The recently published LAURA study found a remarkable improvement in EGFR mutated stage IIIA, IIIB and IIIC patients when treated with consolidating Osimertinib. While in the Osimertinib group PFS reached 39.1 months (HR 0.16) in the control group PFS only reached 5.6 months. A similar study for ALK fusion NSCLC is ongoing.

3.5.2 Oligometastatic disease (OMD) – stage IV A

The definition of oligometastatic disease is still debated. The UICC/AJCC tnm 8th edition defines stage IV A as one single solitary extra thoracic metastasis (M1b) and refers to this as oligometastatic disease (OMD). If additional extra thoracic metastases are present, these are than classified as M1c and stage is changed to IVB. This stage excludes curative treatment[74] However, several studys that investigated potential benefit of curative treatment in OMD enrolled patients with more than one extrathoracic metastases.[84] Finally the EORTC lung cancer group published a consensus statement based on previous studies that set the maximum number of metastases for OMD to be 5 in 3 or less different organ systems.[85]

In this setting some patients may benefit from curative treatment, however all cases should be discussed in a multidisciplinary tumor board and decided on a case-to-case basis. Furthermore, all patients must receive complete staging including PET-CT and MRT to exclude brain metastases. If OMD patients are operable (local stage IIIA) and metastases can be treated by ablative therapy curative treatment may be planned. [86,87]

In one study including 49 patients with up to 2 extra thoracic metastases curative treatment showed remarkable improvement in OS (41.2 months vs 17 months) when compared to palliative treatment.[88] Contrary to these findings the recent presented data of prospective NRG-LU002 trial (NCT03137771) found no statistically significant difference in OS. The study enrolled over 216 patients, with up to 3 metastatic sites after induction immunochemotherapy. Interestingly the lenient inclusion criteria lead to a relatively heterogeneous cohort, with patients enrolled that had up to 25 metastases before systemic treatment. However, the majority of patients only had less than 3 metastases at diagnosis. Still the study is the largest OMD study to date, therefore these results are not easily dismissed. Additional studies are necessary to answer the question of optimal treatment in OMD.

In the meantime, treatment decisions for patients in the oligometastatic disease should be made in a multidisciplinary tumor board on a case-by-case basis. Mediastinal staging should include EBUS-TBNA in suspect lymph nodes. All patients should receive systemic induction treatment before curative treatment should be evaluated. The chemotherapy should contain cisplatin if possible. Furthermore, signs of progression should be monitored closely, and patients may benefit from earlier staging than usually performed. If after induction therapy at least stable disease is reached and the tumor is operable, anatomical resection and mediastinal lymph node dissection should be performed and all additional metastases undergo ablative treatment as soon as possible afterwards.

The role of immunotherapy and targeted therapies in OMD is unclear and may be answered by future studies. Since both therapy options are approved in stage IV prescription is not off label and could be recommended although duration of treatment is unclear at the moment.[87]

3.5.3 Systemic disease – Stage IV B in presence of targetable driver mutations

In stage IVB treatment options depend on patients' performance status, tumor molecular genetics and PD-L1 expression. If performance status is equal or worse than ECOG 3, patients do not benefit from palliative systemic treatment in most cases. However, there may be some exceptions. For example, patients with driver mutations and approved first line TKI treatment should receive treatment even in reduced performance status up to ECOG 3. These mutations include EGFR, ALK, BRAF V600, ROS1, NTRK, RET. Furthermore, the more recently published IPSOS study included driver negative NSCLC patients with reduced performance status (including ECOG III) [89]. These patients received mono checkpoint inhibitor therapy with atezolizumab instead of immunochemotherapy. Although the group of ECOG III patients was small, this study enables a new therapy option in this subgroup of patients. However, most patients with reduced performance status (ECOG III-IV) do not benefit from palliative systemic treatment and receive only symptomatic palliative care. In patients with breathlessness, pain or anxiety morphines and other drugs are commonly prescribed to reduce suffering. Even palliative radiotherapy may be indicated to control treatment refractory pain caused by metastases. If optimal care cannot be provided by the family at home referral to hospice is recommended.

3.5.3.1 EGFR

EGFR mutations include L858R, Del 19 and Exon 20 insertion, as well as rare EGFR mutations. For L858R mutation, Del 19, sensitive Exon 20 insertion and rare EGFR mutations Osimertinib, Afatinib and Dacomitinib are approved in Europe, with Osimertinib showing the longest PFS and overall

survival.[90] Older first and second generation EGFR TKIs are approved but not prescribed in clinical routine due to their higher complication rate. Therefore, first line treatment for EGFR mutated NSCLC patients consisted of Osimertinib in most cancer centres. Recent studies like FLAURA 2 found additional benefit if patients received chemotherapy in addition to Osimertinib, however most patients are hesitant to undergo chemotherapy if there are treatment alternatives.[91] With the combination of amivantamab and Lazertinib there is a another first line treatment option, however this therapy regimen is still not approved in Austria.[92] Unfortunately, after progression under Osimertinib only a limited number of treatment options remain. One option is the combination of amivantamab and lazertinib as shown in the Mariposa 2 trial.[93] Another substance that will be approved in the future is ivonescimab in combination with chemotherapy.[94] To this day only chemotherapy is approved after progression. If afatinib or dacomitinib are used as first line options Osimertinib may be used in second line after progression in T790M positive patients.

3.5.3.2 ALK

For patients with ALK fusions and metastatic disease Crizotinib, Ceritinib, Alectinib, Brigatinib and Lorlatinib are approved in the first line setting. If treatment is started with other ALK inhibitors sequencing with Lorlatinib is approved, however it must be pointed out that not all patients receive second line treatment. Current guidelines recommend treatment with another ALK-TKI after progression. However, a recent update from the CROWN study showed an extraordinary PFS of 60% after 5 years under treatment with Lorlatinib a substantial improvement when compared to other first line ALK TKIs. Unfortunately, Lorlatinib is also the substance with the highest rate of adverse events. Still based on the CROWN data Lorlatinib is standard of care in the first line setting of most cancer centres.[95] After progression only chemotherapy remains as treatment option.

3.5.3.3 ROS-1

ROS-1 translocations are rare and are found in up to 1-2% of all NSCLC patients. In the first line setting crizotinib and entrectinib are approved. Both substances show comparable response rates and PFS, however entrectinib is superior in patients with CNS metastases. After progression rebiopsy should be performed and if G2032R mutation is excluded patients might benefit from lorlatinib. However, this substance is not approved in this indication.

3.5.3.4 BRAF V600

BRAF V600 is another rare mutation and accounts for up to 2% of NSCLC patients. Treatment of choice is the combination of the BRAF inhibitor dabrafenib and the MEK inhibitor trametinib. This combination may be used in first or second line after immunochemotherapy. [96] When compared to chemotherapy the TKI combination is associated with comparable PFS but less adverse events.

3.5.3.5 NTRK

NTRK gene fusions (NTRK1, NTRK2, NTRK3) are a very rare subset of NSCLC patients with an incidence of 0.1 to 0.3%. Available TKIs are larotrectinib and entrectinib. Both show reliable response and PFS for NTKR fusion gene-positive tumors and are approved by EMA. In progression chemotherapy is the best option. The benefit of immunotherapy is still unclear.

3.5.3.6 C-met-alterations

C-met alterations are common in NSCLC and are found in adenocarcinoma and squamous cell carinomas. Most important c-met alterations are MET amplification (1-6%), MET exon 14 skipping mutations (1-6%) and MET fusions (below 1%). For c-MET exon 14 skipping mutations there are two newly approved substances, namly tepotinib and capmatinib. Capmatinib reaches higher response rate (68% vs 45%) and higher PFS (12,5 vs 7,9 months) although, direct comparison between trials are not appropriate.[97,98] Capmatinib had some therapeutic activity in tumors with MET amplification and high copy number of more than 10 (response rate 40% median PFS 5,5 months). If Tepotinib also has activity in this space is unclear.

3.5.3.7 RET fusions

Rearranged during Transfection (RET) fusions are very rare and are detected in 1-2% of NSCLC patients. Similar to EGFR and ALK mutations they are far more common in young patients with non- or little smoking history and adenocarcinoma histology. With selpercatinib and pralsetinib there are two highly effective, first line approved substances. Both achieved response rates of up to 80%.[99]

3.5.3.8 KRAS mutations

KRAS mutations are present in approximately 30% of NSCLC cases. The most common KRAS variants in NSCLC are KRAS G12C (53%), G12V (27%), G12D (6%), G12A (~6%) and G12S (~4%). Based on the Codebreak 100 trial Sotorasib is approved for KRAS G12C mutated NSCLC after failure of first

line treatment. The study found a response rate of 37.1% and median PFS of 6.3 months. Recently the Krystal phase II study was positive for adagrasib when compared to second line treatment in the KRAS G12c mutated metastatic NSCLC. The study found an objective response rate of 42.9% and a median PFS of 6.5 months. Based on these results this study will lead to approval of adagrasib in the future.

3.5.3.9 Her2

Human Epidermal Receptor 2 (HER2) is mutated in approximately 3% of NSCLC, while overexpression is found in around 30% of NSCLC cases. Unfortunately, at the time of publication Her2 overexpression are non-targetable in lung cancer. Contrary to other driver mutations like EGFR and ALK, Her2 mutations are not linked to adverse events or weak response under ICIs.[100] In NSCLC trastuzumab-deruxtecan, an antibody-drug-conjugate, is approved for treatment of NSCLC with Her2 mutation.[101] After progression under first line therapy, the combination resulted in PFS of 8.2 months and OS of 17.8 months.[102] This therapy regimen however is associated with a high number of interstitial lung disease, which occurred in up to 26% of patients.

3.5.4 Systemic disease without targetable driver mutations

In absence of targetable driver mutations, the treatment regimen of metastasised NSCLC is based on PD-L1 expression. In patients with PD-L1 expression of below 50% immunochemotherapy is recommended, while patients with PD-L1 expression of 50% or above should receive mono-immunotherapy. However, if the treatment with immunochemotherapy in this subgroup of patients may be beneficial is still unclear. A recent retrospective analysis of the FDA found no statistically significant effect on overall survival for additional chemotherapy. However, the combination therapy was associated with a small but statistically significant advantage in progression-free survival.[103] As seen in previous studies older patients did benefit more from mono-immunotherapy as adverse effect of chemotherapy increase with age. Interestingly, gender had an impact on outcome as well. The study found that female patients benefited less than male patients from mono-immunotherapy. Another important subgroup were non-smokers. These patients benefited more from chemoimmunotherapy, which may be caused by a higher number of occult driver mutations. However, these observations should be confirmed by prospective studies before guiding treatment decisions. Still there is broad consensus that patients with high tumor burden, fast growing tumors of wearing tumor associated symptoms should be evaluated for chemoimmunotherapy.

For treatment of NSCLC patients with PD-L1 expression of 50% and above mono-immunotherapy with Pembrolizumab (Keynote 042), Atezolizumab (Impower 110) or Cemiplimab (Empower-Lung-01) is approved by the EMA. All substances were tested against platinum based doublet chemotherapy and reached comparable improvement in PFS and OS. [104–106]

In NSCLC patients with PD-L1 expression below 50% several chemoimmunotherapy combinations based on histology are available. In non-squamous cell carinoma patients platinum-based chemotherapy in combination with immunotherapy lead to approval of pembrolizumab (Keynote 189), atezolizumab (Impower 130) and cemiplimab (Empower-Lung-03).[107–109] Study outcomes of these drugs were comparable with each other. Keynote 189 found a median OS of 22.0 months (HR 0,56), Empower lung 3 21.9 months (HR 0,71) and impower130 18,6 months (HR 0,79).

Another approved combination in NSCLC is the Impower150 study. These patients received platinum and pemetrexed chemotherapy plus atezolizumab and bevacizumab. The study found prolonged OS (median OS 5,5 months - HR 0,78) and PFS. When compared to previously mentioned therapy regimen this is the only approved ICI treatment option for patients with driver mutations as EGFR, ALK and ROS-1 patients were not excluded in this study. However more recent publications dispute this result. A recent meta-analysis examined 6 publications with EGFR mutated NSCLC patients (KEYNOTE-789, Impower150, Impower151, ORIENT-31, ATTLAS and CheckMate-722) and found no statistically significant improvement in OS.[110] Still no other ICI treatment option for NSCLC with driver mutations is approved.

For squamous cell NSCLC and PD-L1 expression below 50% platinum-based chemotherapy in combination with pembrolizumab vs platinum-based chemotherapy was analysed in the Keynote 407 study. Overall, statistically significant improvement in OS and PFS of 4,6 (HR 0,63) and 1,6 months (HR 0,56) in the pembrolizumab group, which lead to approval. Unfortunately, subgroup analysis of PD-L1 negative patients found no benefit when treated with pembrolizumab.

Another approved treatment regimen was assessed in the checkmate 9LA study. Patients received a combination of ipilimumab 1mg/kgKG and nivolumab 360mg with platinum-based chemotherapy for 2 cycles. After 2 cycles chemotherapy is discontinued and immunotherapy combination continued for 2 further cycles. This study found a significant prolongation of OS (15,6 vs 10,9 months) when compared to chemotherapy alone. A potential advantage of this regimen is the reduction in chemotherapy which may be relevant in older or frail patients. Interestingly a recent subgroup analysis found potential benefit in PD-L1 negative squamous cell carcinoma (HR 0,48) when compared to PD-L1 positive or non-squamous cell carcinoma NSCLC patients (HR 0,70). [111] Another subgroup that benefited

disproportionately were patients with stable CNS metastases, which had a median OS of 19.9 versus 7.9 months in the control group [HR 0.47 (95% CI 0.31-0.71). However, the combination of PD-L1 and CTLA4 checkpoint inhibitors leads to higher rates of adverse events.

Most recently results from the POSEIDON study lead to approval of tremelimumab 1mg/kg body weight, another CTL-A4 inhibitor, in combination with durvalumab 1500 mg and platinum-based chemotherapy in metastasised NSCLC.[112] After completion of 4 cycles of immunochemotherapy and confirmed stable disease first line therapy maintenance should be offered. Maintenance therapy depends on histology and therapy regime but may include pemetrexed until progression for non-squamous cell carinoma patients: pembrolizumab every 3 or 6 weeks over 35 cycles, pemetrexed pembrolizumab combination every 3 or 6 weeks for up to 35 cycles, cemiplimab for up to 2 years in empower 01 and until progression in empower 03, nivolumab ipilimumab for up to 2 years or durvalumab with optional chemotherapy until progression. In absence of targetable driver mutations second line therapy options are mostly limited to chemotherapy. In non-squamous cell carinoma patients second line treatment options are docetaxel, with or without Nintedanib or Ramucirumab. Addition of Nintedanib, a multi-kinase inhibitor, to docetaxel lead to an increase in median OS in non-squamous cell carinoma NSCLC patients of 12,6 months compared to 10,3 months in the control group. (HR 0,83) in the Lume-Lung-01study.[113] This benefit was not found for SCC patients. The REVEL study evaluated the use of Ramucirumab, a monoclonal antibody that targets VEGFR-2, in addition to docetaxel in second line NSCLC. Ramucirumab led to an increase in median of 10,5 months vs 9,1 months (HR 0,86).[114] Both substances are approved for second line treatment. In SCC LUX Lung 08 showed superior efficacy of Afatinib over Erlotinib with a median OS of 7.9 vs 6.8 months. Both substances were superior to chemotherapy.[115] Second line treatment with ICIs like Pembrolizumab, Atezolizumab and Nivolumab are approved based on Keynote 033, OAK and the Checkmate 17 study respectively.[116–118] However, because most patients receive ICIs in the first line continuation therapy with ICIs in the second line is not reasonable in most patients. Still, some patients may benefit from retreatment with checkpoint inhibitors.[119] Best response rates were seen in patients that progressed after long duration of treatment with ICIs or discontinuation of ICI treatment after maintenance therapy was finished. In these settings objective control rate reached up to 75% by retreatment.[120] Whether substance change has impact on response is unclear. At least one publication found no statistically significant impact of substance change.

3.6 Immunological biomarkers

The treatment landscape for non-small cell lung cancer (NSCLC) has undergone a significant transformation with the advent of immune checkpoint inhibitors (ICIs). Central to the success of these therapies is the identification and utilization of immunological biomarkers, which guide clinical decisions and predict patient responses. To date, the only biomarker routinely used in clinical practice is the PD-L1 expression. However, PD-L1 expression remains an imperfect predictor of response to immunotherapy. Additionally, PD-L1 expression appears to undergo rapid changes that are not yet well understood, which may have implications for therapy. Furthermore, PD-L1 expression may not predict response to checkpoint inhibitors targeting molecules other than PD-L1/PD-1, such as CTLA-4. Although research in this field is gaining momentum, a more robust biomarker for ICI treatment remains elusive. In the following, we discuss the most promising immunological biomarkers in the treatment of lung cancer.

3.6.1 PD-L1 Expression

PD-L1 (Programmed Death-Ligand 1), also called CD274 or B7-H1, is expressed by tumor cells and some immune cells. It plays a key role in modulation of the immune response. In healthy individuals PD-L1 signalling is maintaining immune homeostasis by deactivation of PD-1 presenting immune cells. Unfortunately, by expression of PD-L1 tumor cells can suppress anti-neoplastic immunity. PD-L1 (programmed death-ligand 1) is measured through immunohistochemistry (IHC). For this procedure a tumor tissue sample is fixed in formalin and embedded in paraffin. It is then sliced into thin sections. In the next step the tumor slide is then stained using PD-L1 binding proteins. In Graz Ventana PD-L1 (SP263) immunohistochemistry assay is used for this purpose. Finally, a pathologist examines the stained tissue and quantifies PD-L1 expression. The percentage of cells showing PD-L1 staining is reported, often as a "Tumor Proportion Score" (TPS) or "Combined Positive Score" (CPS). Higher PD-L1 TPS in tumors are associated with worse prognosis in NSCLC when compared to PD-L1 negative patients. When treated with ICI however, patients with higher PD-L1 expression show longer PFS and OS than PD-L1 low or negative patients[121,122]. That’s why the approval of ICIs for the treatment of metastatic NSCLC is often linked to PD-L1 expression. Still, even PD-L1 negative patients seem to benefit from PD-L1 or PD-1 checkpoint inhibition usually in up to 20% of cases depending on the tested checkpoint inhibitor.[123,124] This leads to the fact that PD-L1 expression alone is unreliable in predicting response to immunotherapy or the duration of its effectiveness[123]. The poor accuracy of PD-L1 immunohistochemistry as a biomarker for ICI treatment is probably the result of multiple variables including factors like state of the immune system overall, quantity of tumor infiltrating immune-cells, and activation of suppressive immune pathways (eg, IDO, FoxP3+ regulatory T cells,

and lymphocyte-activation gene 3 [LAG3]) that lead to tumor immune escape. Another important reason why PD-L1 expression fails to predict response to treatment in some cases and is accurate in others is dynamic PD-L1 expression change and tumor heterogeneity as discussed below. [125]

3.6.2 Tumor mutational burden (TMB)

TMB refers to the total number of somatic mutations per megabase (Mb) of the coding region of a tumor's DNA. It quantifies the number of mutations present within the tumor genome. Tumors with a high TMB have more genetic alterations, which can lead to the production of more neoantigens. The immune system relies on recognizing these neoantigens to identify and attack cancer cells. A higher TMB therefore increases the likelihood that the tumor will present neoantigens on its surface, making it more visible to the immune system. When treated with checkpoint inhibitors, such as anti-PD-1 or anti-PD-L1 therapies, the presence of these neoantigens can enhance the immune response. Studies have shown that patients with tumors that have a high TMB tend to respond better to checkpoint inhibitors compared to those with low TMB.[126,127] Still TMB alone is not able to predict response to checkpoint inhibitors reliably.[128] That's why it is only used in conjunction with other biomarkers like PD-L1 immunohistochemistry or microsatellite instability (MSI) to predict response to treatment. In treatment of lung cancer, there is no recommendation for routine use of this biomarker up to this point.[129]

3.6.3 Microsatellite instability (MSI) and deficient mismatch repair (dMMR)

Microsatellites (MSs) are short DNA segments that are usually not longer than 6 nucleotides. These Microsatellites are located in both gene and intergene areas and repeat throughout the genome.[130,131] MS instability (MSI) occurs when the genome gains or loses these repeating segments. Usually, these errors are corrected by a DNA repair system called mismatch repair (MMR). The proteins that are part of the MMR system involve among others MLH1, MSH2, PMS2 and MSH6). If this MMR system is inactivated by mutations in genes coding for these repair molecules this results in an inability to repair errors that occur during DNA replication. These errors tend to occur in MS regions, therefore tumors with these errors are classified as MSIs. The result of ineffective MMR is a higher number of mutations and neoantigens. As these errors tend to occur predominantly in MS regions, tumors with such errors are regarded as having MSI-H. Due to deficient MMR, both the number of mutations and neoantigens are higher, which in turn leads to activation of antineoplastic immunity. Le and colleagues investigated the response to pembrolizumab in metastatic cancer with or without MSI. They found response in 78% of non-CRC dMMR patients but no response in MMR intact patients.[132] Based on further studies that

confirmed these findings for solid tumors in a larger cohort, the FDA approved pembrolizumab for MSI instability irrespectively of cancer type.[133] Unfortunately, the prevalence of MSI/ dMMR is low in NSCLC. In a cohort of over 12000 tumors, Le and colleagues found rates of MSI/ dMMR of higher than 10% in CRC, while in NSCLC it was below 2%.[130] Therefore, MSI as predictive tumor marker in NSCLC is of limited use.

3.6.4 Immune-modulating genetic alterations

3.6.4.1 Driver mutations

NSCLC with classical driver mutations like EGFR, ALK, ROS1 and RET show almost no response to ICIs. These mutations are more common in younger patients, that are light or never smokers, which results in a low mutational burden and therefore lower likelihood of immunogenicity.[134,135] BRAF V600 and MET Exon 14 mutations are more common in smokers and may respond to checkpoint inhibition, but response varies between studies.[135] In KRAS mutated NSCLC response to ICIs seems to be unimpaired when compared to KRAS wildtype. However, in combination with co-mutations like STK11 or KEAP1 downregulation of anti-neoplastic immunity and therefore less response to ICIs has been described.[136–138]

3.6.4.2 STK11 Mutation

STK11 is a tumor suppressor gene that influences diverse regulating cellular processes including cell metabolism, growth and polarity. Loss-of-function STK11 mutation in found in about 10% to 15% of NSCLC and more prevalent in lung adenocarcinoma and western populations.[139] In previous publications STK11 mutation was associated with reduced expression of PD-L1, reduced tumor-infiltrating cytotoxic CD8 positive T lymphocytes and higher rates of immune suppressive cells. Therefore, these tumors are described as immunologically cold tumors by some authors. [140] This impairment of anti-neoplastic immunity seems to be associated with treatment failure under checkpoint inhibitor therapy.[137,141] A recent study with over 200 STK11 mutated NSCLC patients found no additional benefit of ICI when compared to chemotherapy alone.[142] Furthermore, several studies found reduced overall survival in STK11 mutated NSCLC patients.[143] However, some publications found contradictory results. A subgroup analysis of KEYNOTE 042 found no influence of STK11 mutation status on ICI treatment response[144] In a prospective phase 2 trial check point inhibitors were even more effective among patients harbouring the STK11 mutation. Recently, several exploratory analyses of phase 3 NSCLC trials suggested that ICB improved outcomes compared with chemotherapy regardless of the STK11 status.[112,145,146]. The reason of these conflicting results is still unclear, however some authors hypothesized that co mutations like KRAS, KEAP-1, tp53 as well as the STK11

phenotype might play a role in ICI treatment response.[137,147,148] In conclusion, the role of STK11 mutation as a biomarker for NSCLC is debated intensively and has limited therapeutic consequences in daily clinical practice.

3.6.4.3 KEAP-1

Kelch-like ECH-associated protein 1 (KEAP1) is an important regulator of NRF2 transcription factor, which regulates the cellular response to oxidative stress. KEAP-1 loss is found in about 20% of lung adenocarcinomas and is often associated with STK11mutation. This may be due to the fact that an increase in NRF2 activity can reduce the oxidative stress induced by STK11 loss, thereby selecting for KEAP1 mutated tumors.[149] KEAP1 mutated NSCLC tumors and have been linked to rapid progression and reduced response to chemotherapy, targeted therapy and radiation.[150,151] The role of immunotherapy in KEAP1 tumors is still unclear. A recent retrospective database analysis including 69 KEAP1 mutated NSCLC patients found a higher TMB in these patients. Still patients with wild type KEAP1 had longer OS than mutated KEAP1 patients, which suggests that KEAP1 mutation may be unfavourable for ICI treatment.[152] These findings were confirmed by Manelli and colleagues. [153] Subgroup analysis of IMPOWER 150 and POSEIDON study found lower PD-L1 expression in KEAP1 mutated tumors and reduced OS irrespective of ICI treatment.[112,145] Similar to STK11 impact of KEAP1 on clinical routine is still unclear.

3.6.5 LAG3

LAG3 (Lymphocyte Activation Gene-3) is an emerging immune checkpoint molecule that plays a significant role in regulating T cell function and maintaining immune homeostasis. Its role as a biomarker for immunotherapy in non-small cell lung cancer (NSCLC) is an area of active research. It is expressed on activated T cells, regulatory T cells (Tregs), natural killer (NK) cells, and some B cells. It functions as an inhibitory receptor, similar to PD-1 and CTLA-4, and negatively regulates T cell proliferation, activation, and cytokine production. Activation of LAG3 is therefore associated of t cell exhaustion and poorer prognosis. Lag3 is often co-expressed with PD-1 on exhausted T cells within the tumor microenvironment. This co-expression indicates a more profound level of immune exhaustion and may suggest that tumors expressing high levels of Lag3 are more resistant to PD-1/PD-L1 inhibitors. Therefore, Lag3 could serve as a biomarker to identify patients who may benefit from combination therapies targeting both PD-1 and Lag3.

In the Relativity 047 study, the anti-Lag3 antibody relatlimag was assessed in combination with nivolumab vs nivolumab alone in melanoma patients. The study found a significant improvement in PFS when compared to PD-1 inhibition alone.[154] Although there are no clinical trials in NSCLC Lag3 targeting immunotherapy has the potential to expand the arsenal of check point inhibitors. In conclusion, LAG-3 has the potential to be an important biomarker in the immunotherapy of NSCLC, particularly as a predictor of response to combination checkpoint inhibitor therapies. However, further clinical research is needed.

3.6.6 TILS

The presence and quantity of Tumor-Infiltrating Lymphocytes (TILs) both in surgical specimen and in biopsy samples have been investigated as potential prognostic biomarker for various cancers, including non-small cell lung cancer (NSCLC). TILs primarily consist of T cells (CD4+ helper T cells and CD8+ cytotoxic T cells), which play crucial roles in recognizing and attacking tumor cells. Numerous studies have shown that a higher density of TILs in NSCLC is associated with better clinical outcomes, including improved overall survival and disease-free survival. Kilic et al found that higher TILS density was associated with lower risk of recurrence and survival after resection in early stage lung cancer.[155] These findings were confirmed by several similar studies in locally advanced stages. [156,157] Still several publications found response to check point inhibitors even in patients with TIL-low-tumors as well as lack of response in TIL-high tumors.[157,158] Gide and collegues investigated T-cell subpopulation from biopsies in melanom responders compared to non-responders treated with checkpoint inhibitors.[158] They found that high density TIL ICI non responding tumors show overexpression of IDO1, ICOS and TIGIT that are associated with upregulation of regulatory t cells and suppression of anti-neoplastic immunity.[158] Therefore TIL density in tumor tissue alone is insufficient as sole biomarker for checkpoint inhibitor response. Further research into TIL subpopulation is necessary and may lead to development of robust biomarkers in the future.

3.6.7 Regulatory T cells (TREG-cells)

Regulatory T cells (Treg cells) are a specialized subset of T cells crucial for maintaining immune balance by suppressing excessive immune responses and preventing autoimmunity. Characterized by markers like CD4 and FOXP3, Treg cells modulate the immune system by inhibiting the activity of other immune cells and reducing inflammatory cytokine production. This is facilitated by release of suppressive cytokines (such as IL-10 and TGF-β) which leads to inhibition of the activity of immune cells like cytotoxic T cells.

Unfortunately, while necessary to prevent autoimmune disease, in cancer suppression of immune response leads to reduced effectiveness of anti-tumor immunity. Therefore, Treg cells may be both a interesting biomarker für ICI treatment, as well as a possible target for immune modulating therapies.[159]

Recently the CONTINUUM trial evaluated the predictive value of circulating Treg cells as biomarker for response to ICI treatment in nasopharyngeal cancer.[160] The authors were interested in the specific subsets of regulatory t cells in patients that responded to ICI treatment in comparison to those that did not respond. They found that a specific subset of Treg cells called Ki67+ Tregs was associated with early progression and concluded that this cell may be a future biomarker or target for immunomodulating therapy.[160]

However, research into Treg cells is still ongoing. At the time of writing analysis of Treg cells is not recommended for clinical routine use.

3.6.8 The gut microbiome

The human gut is colonized by countless, mostly symbiotic microorganisms collectively referred to as the gut microbiome. These microorganisms are of immense importance for human homeostasis. The gut microbiome is essential for the development and function of both the innate and adaptive immune systems, influences the permeability of epithelial barriers, and, through its synthesis of vitamins and amino acids, constitutes indispensable components of the human body. Therefore, it should come as no surprise that the microbiome also has a clinically relevant effect on the efficacy of oncological therapies.[161,162] Of particular interest in this regard, is the interaction of the microbiome with the immune system and its influence on therapy with immune checkpoint inhibitors (ICIs). [162,163] Treatment with ICIs is now an essential component of many modern neoadjuvant, adjuvant, and palliative oncological therapy concepts. However, 60 to 80% of treated patients do not respond to ICI therapy. Unfortunately, despite immense scientific interest, there is currently no biomarker that can reliably predict response to ICIs.[164] A multitude of studies in both mouse models and humans have now demonstrated a correlation between the composition of the gut microbiome and the response to various ICIs. Thus, the gut microbiome could be a source of potential biomarkers for the future.[165] In this context both preclinical models and studies in patients have now sufficiently demonstrated that the relative composition of the microbiome is suitable as a biomarker for predicting response to immunotherapies.[162,166,167] For example, Routy and colleagues showed in 2018 that mice kept under germ-free conditions and with a completely depleted gut flora due to antibiotic treatment exhibited hardly any antineoplastic immune response following administration of PD-L1 inhibitors. However, after oral administration of Akkermansia muciniphila, the number of tumor-infiltrating cytotoxic T cells

increased, and the tumor volume significantly decreased compared to the placebo group. Routy and colleagues further substantiated these findings by performing stool transplants from immunotherapy responders with non-small cell lung cancer and renal cell carcinoma into mice, though only in a small cohort.[167] In a prospective study involving 338 patients with non-small cell lung cancer, Derosa et al. demonstrated that both the detection and relative abundance of A. muciniphila were associated with a favorable response to immunotherapy, validating the results of Routy and colleagues in a larger cohort.[163] A study also demonstrated a correlation between α-diversity (a measure of the number of different species in a microbiome) and response to immunotherapies.[168] For a long time, this was considered the most relevant factor influencing the effectiveness of immunotherapy. However, this finding could not be confirmed by larger subsequent studies. A series of recent meta-analyses also failed to show a correlation between response and microbiome diversity. Thus, it must currently be assumed that, contrary to initial beliefs, microbiome diversity alone does not influence the response to immunotherapy, and that the initial results were likely due to methodological factors.[166,169,170] How exactly colonisation of specific microorganism in the gut leads to specific outcomes in treatment with ICIs is the subject of intense research. A recent publication demonstrated that some Enterococcus species produce specific clusters of peptidoglycan hydrolases, such as secreted antigen A (SagA). In mouse models, these were associated with increased T-cell infiltration into tumor tissues and a significantly greater reduction in tumor mass. Interestingly, similar effects could be achieved through the probiotic administration of SagA as well as SagA-associated synthetic muropeptide cleavage products (MDP).[162,171] SagA-related hydrolases have also been detected in other commensal species such as Lactobacillus and Bifidobacterium bifidum. Another key mechanism involves the activation of the STING pathway by microbiome-derived products such as c-di-AMP, which leads to enhanced production of type I IFN-γ by monocytic phagocytes both locally and systemically. Type I IFN-γ plays an essential role in the immune system's tumor surveillance. C-di-AMP and related metabolites are produced by commensals such as Akkermansia muciniphila, among others.[172] In conclusion, the gut microbiome holds significant promise as a biomarker for predicting and enhancing the response to immunotherapy in cancers like NSCLC. Its influence on the immune system suggests that modulating the microbiome could improve the efficacy of immune checkpoint inhibitors. However, further research is needed to fully understand the mechanisms at play, develop standardized testing methods, and explore therapeutic interventions targeting the gut microbiome.

3.6.9 Future prospect of immunological biomarkers and aim of this study

Although multiple immunological biomarkers are of high clinical interest and may play a role in the future in current clinical practise the treatment of NSCLC in absence of driver mutations is still based

primarily on PD-L1 expression levels. However, PD-L1 expression still is no accurate biomarker for ICI response. Therefore, an improved understanding of potential influencing factors of PD-L1 expression may help to improve diagnostic accuracy and may impact clinical outcome in NSCLC patients. To answer this research question, we compared PD-L1 expression in initial biopsy, surgical specimen and rebiopsy in case of relapse and evaluated potential influencing factors such as neoadjuvant and adjuvant treatment or tumor heterogeneity.

4 Material and Methods

4.1 Study design and patients

We evaluated all patients who underwent surgery for NSCLC between December 2015 and December 2020 at the Medical University of Graz (figure 1). After surgery patients were observed. The Patients that experienced disease relapse and underwent a rebiopsy until January 2023 were included in this study. All patients with synchronous metastatic disease or driver mutations that do not allow for ICI in the first line setting had to be excluded.

4.1.1 Inclusion criteria

Patients that fulfilled the following criteria were included:

- NSCLC with localized disease (stage I-III)
- Curative surgery performed
- BRAF and KRAS mutation allowed

Stage of the disease was determined using the 8th edition UICC classification for NSCLC[77].

4.1.2 Exclusion criteria

Patients that fulfilled the following criteria were excluded:

- Synchronous metastases
- Driver mutations that do not allow the use of ICIs in the first-line setting (EGFR, ALK, ROS, RET and NTRK)

4.1.3 Exclusion of second primary lung tumors

In order to confirm diagnosis every case was discussed in a multidisciplinary tumor board. In cases of pulmonary relapse, secondary primary was either excluded by clinical or histological criteria. If no definitive exclusion was possible a molecular analysis by next generation sequencing (NGS) was performed to rule out a secondary primary of the lung. Using these criteria, secondary primary tumors were excluded for all patients in our study cohort.

For all patients clinical data about age, sex, smoking status, Eastern Cooperative Oncology Group (ECOG) performance status, histology, clinical preoperative and postoperative stage of disease, and neoadjuvant or adjuvant treatment modalities were gathered.

4.1.4 PD-L1 Expression

PD-L1 expression of preoperative biopsy samples, surgical specimen and rebiopsy was documented. In cases were PD-L1 expression status was not available at any of these three time points, it was assessed retrospectively. The IHC assay used for PD-L1 Expression was the Ventana PD-L1 (SP263), which was used in all cases. PD-L1 expression results were grouped in 3 score groups. These score groups were PD-L1 without expression, intermediate PD-L1 expression 1%–49% and high PD-L1 expression ≥50%.

4.1.5 Tumor Heterogeneity

To investigate the possible influence of tumor heterogeneity on PD-L1 expression change, we re-assessed the surgical specimens of patients who changed PD-L1 expression score group every single time. This was the case in five of 72 patients. To answer this question, we evaluated the whole remaining surgical tumor block. In all patients between six to eight slides could be analysed for PD-L1 expression.

4.1.6 Primary Endpoint

The primary endpoint was the change in absolute PD-L1 and PD-L1 score group between the different time points.

4.1.7 Statistical analysis

Statistical tests were performed using the IBM SPSS Statistics version 28. Comparison of variable distribution between samples with and without PD-L1 score group change were compared using Chi-Square test. Statistical significance level was defined as 5% (p=0.05). If data were missing, they are shown in separate columns in total numbers and percent. In order to evaluate the impact of neoadjuvant or adjuvant treatment and location of rebiopsy on PD-L1 expression change Mann-Whitney-U-Test was performed. To exclude the impact of timing of rebiopsy on PD-L1 expression change Spearman correlation model was used.

4.2 Ethical issues and data protecting

The study protocol conformed to the Declaration of Helsinki and was approved by the Ethics Committee of the Medical University of Graz (EK 33-174 ex 20/21).

We saved patient-related data on a firewall-protected and password-saved server of the Medical University of Graz. All data were saved anonymously in Microsoft Office Excel 2016 program and only authorized personnel had access to the server.

5 Results

Between December 2015 and December 2020, 612 patients underwent surgery with curative intent for diagnosed or suspected NSCLC.

However, in a high number of patients surgery led to diagnosis of different malignant diseases than NSCLC. As only NSCLC patients were analyzed those patients (n=110) had to be excluded. In some cases (n=56), synchronous metastatic disease was diagnosed intraoperatively or within days of surgery, which led to exclusion. Furthermore, since driver mutations like EGFR, ALK, ROS-1 and NTRK do not allow for ICIs in the first line, patients with these genetic alterations (n=51) could not be considered for our analysis as well.

In summary, a total of 395 patients with localized NSCLC (stage I-III) were included. Out of these 395 patients 259 patients did not progress to this date, therefore only 136 patients met inclusion criteria. Of these patients only 87 were re-biopsied and could be included for further analysis. Figure 1 shows the procedure of our patient selection.

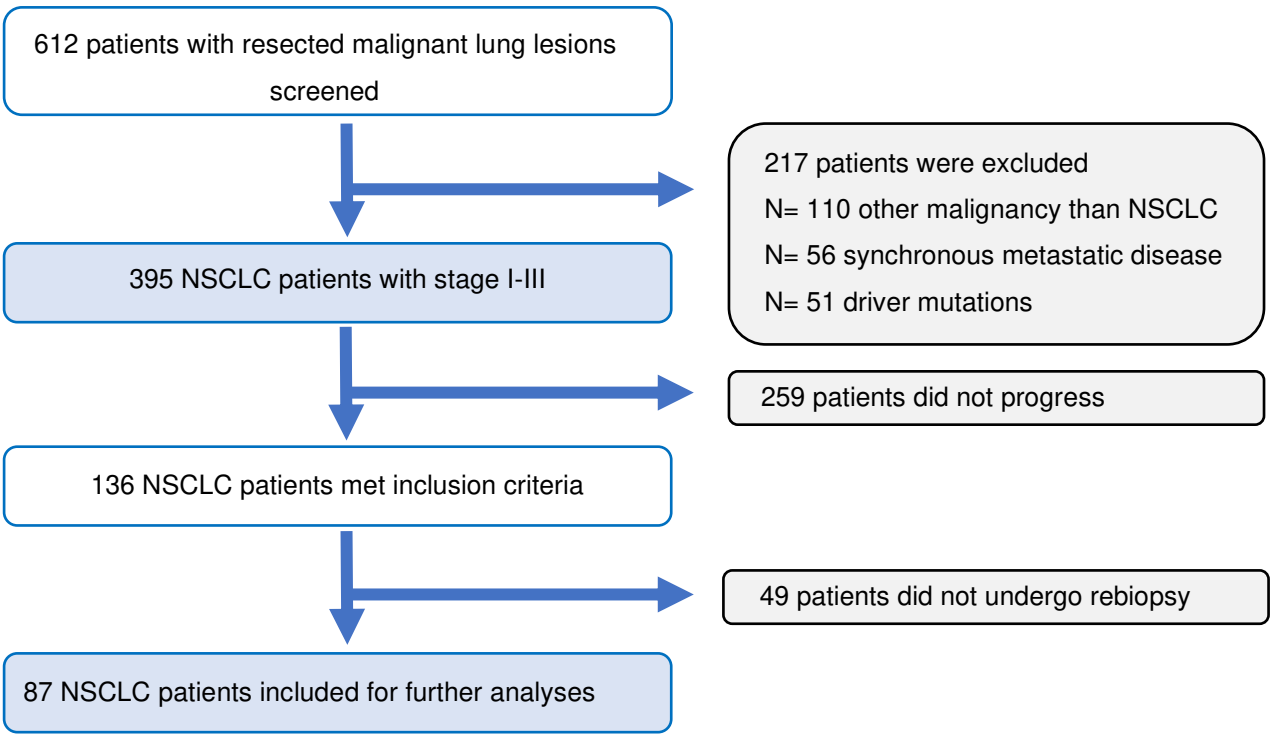

Figure 3 CONSORT *(Consolidated Standards of Reporting Trials) diagram and study design of "Longitudinal analysis of PD-L1 expression in patients with relapsed NSCLC"*[173] *- NSCLC – non-small cell lung cancer.*

5.1 Clinical and demographic characteristics

Table 8. Baseline Characteristics of the Study cohort (N=87)		
	N	***%***
Gender		
Male	52	59.8
Female	35	40.2
Performance Status		
0	32	36.8
1	37	42.6
≥ 2	5	5.7
missing	13	14.9
Smoking		
Never	5	5.7
Current or Former	62	71.3
missing	20	23
Histology		
Adenocarcinoma	52	59.8
Squamous cell carcinoma	32	36.8
Other	3	3.4
Postoperative Tumor Stage		
I	17	19.5

II	35	40.2
III	33	37.9
missing*	2	2.3
Perioperative Treatment		
Neoadjuvant	15	17.2
Adjuvant	25	28.7
Both	4	4.6
Time between surgery and rebiopsy		
< 1 year	32	36.8
≥ 1 year	55	63.2
*2 patients were deemed inoperable during surgery and underwent definitive chemoradiation.		

Table 8 *Baseline characteristics of the study cohort*

As shown in the consort diagram 87 patients formed our study cohort. The median age within the cohort was 63 years (range 44 to 83). The majority of patients were male (59.8%) and current or former smokers (71%). Most patients (78.2%) had stage II and III disease at the time of surgery. Baseline demographic and disease characteristics are detailed in table 1.

5.2 Neoadjuvant and adjuvant treatment

Fifteen patients (17.2%) received neoadjuvant treatment, four patients (4.6%) had neoadjuvant and adjuvant treatment and 25 patients (28.7%) were treated in the adjuvant setting. The patients that did receive neoadjuvant chemotherapy received cisplatin in twelve cases. The rest received carboplatin. Platinbased chemotherapy was combined with Pemetrexed in four, Vinorelbine in 3, Gemcitabine in 3 and Docetaxel in 4 patients. Neoadjuvant chemoradiation was performed in 6 cases. The patients were treated with adjuvant treatment received platin-based chemotherapy in 21 cases. Almost all patients received Cisplatin (n=19). Pemetrexed, Vinorelbine, Gemcitabine and Etoposid were prescribed in three, thirteen, four and one patient respectively. Mediastinal adjuvant Radiotherapy was performed in 4 cases. No patient received perioperative treatment with ICIs as there was no approval at that time in this setting. However, due to participation in the AEGEAN trial one patient received neoadjuvant and adjuvant ICI treatment with Atezolizumab. These results are shown in Table 2.

Table 2. Type of neoadjuvant and adjuvant therapy		
	Number	Percent %
Neoadjuvant chemotherapy	15	100
Pemetrexed	4	26
Vinorelbine	3	20
Gemcitabine	3	20
Docetaxel	4	26
Adjuvant chemotherapy	21	100
Pemetrexed	3	14
Vinorelbine	13	58
Gemcitabine	4	19
Etoposid	1	9
Radiotherapy	10	100
Neoadjuvant	6	60
adjuvant	4	40

Table 9 *Type of neoadjuvant and adjuvant therapy*

5.3 Recurrence and site of rebiopsy

Out of 87 relapsed patients 34 experienced strictly local recurrence (lung or mediastinal lymph nodes) whereas 53 patients had distant failure. Rebiopsy was performed from lung, mediastinal lymph node, bone or soft tissue, brain, pleura, distant lymph node, liver, adrenal gland and pancreas in 33, 13, 12, 10, 7, 6, 3, 1 and 1 patients respectively. The results are shown in Table 3.

Table 3. Type and location of recurrence		
	Number	**Percent %**
Recurrence		
Local	36	41
Distant	51	59
Site of rebiopsy		
Lung	33	37
Mediastinal lymph node	13	14
Bone and soft tissue	12	13
Brain	10	11
Pleura	7	8
Distant lymph node	6	6
Liver	3	3
Adrenal glande	1	1
Pancreas	1	1

Table 10 *Type and location of recurrence*

5.4 PD-L1 expression change between initial biopsy and surgical specimen

In 72 (82.8%) out of 87 patients, preoperative biopsies and matched surgical samples were available for comparison of PD-L1 expression. When divided into clinically relevant groups of PD-L1 expression (0%, 1-49% and ≥ 50%), we found a treatment relevant group change in 25 patients (34.7%). Eleven patients (15.3%) swapped into a higher PD-L1 score group, 14 patients (19.4%) changed into a lower group and 47 patients (65.3%) stayed in the same group. Preoperative tumor stage was stage I in eleven (15.7%), stage II in 27 (38.6%) and stage III in 32 (45.7%) patients. Sixteen (22.2%) out of the 72 patients received neoadjuvant treatment, twelve patients (75%) being treated with neoadjuvant

chemotherapy, three (18.8%) with chemoradiation and one patient (6.2%) with SBRT who finally underwent surgery for disease progression. Response to neoadjuvant treatment was partial response in eleven (68.8%), stable disease in three (18.8%) and progressive disease in two cases (12.5%). We found no statistically significant influence of neoadjuvant treatment on the change of the PD-L1 absolute expression PD-L1 score group in preoperative biopsy and matched surgical specimen (p=0.39, table 4). Even if absolute PD-L1 expression change was considered we found no statistically significant influence (p=0,670, figure 2) Also, the response to neoadjuvant treatment did not show a significant association with a change of the PD-L1 score group (p=0.91, table 4).

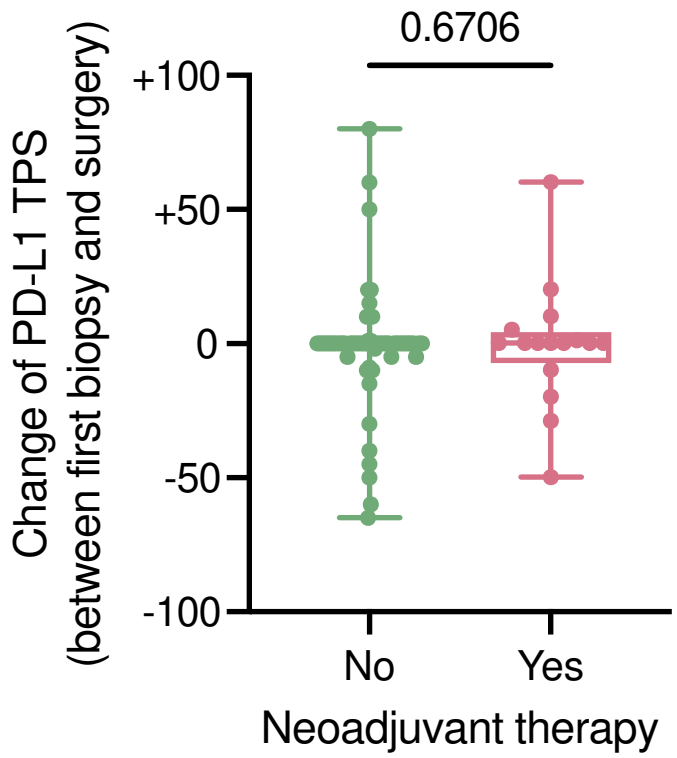

Figure 4 *Absolut PD-L1 expression change between initial biopsy and surgical specimen with and without neoadjuvant chemotherapy*

5.4.1 Influence of neoadjuvant chemotherapy agent and radiation

Neoadjuvant chemotherapy regimen in our cohort consisted of a platinum backbone in combination with either pemetrexed (n=4), vinorelbine (n=3), gemcitabine (n=3) or docetaxel (n=2). We compared PD-L1 expression group change as described above between initial biopsy sample and surgical specimen to examine potential influence on PD-L1 expression. Within the pemetrexed group three patients remained in the same group, one changed in the PD-L1 high group (40 to 100%). Neoadjuvant Vinorelbine was associated with no PD-L1 expression group change in two and one case of PD-L1 expression decrease (50% to 0%). All patients that were treated with gemcitabine showed no PD-L1 expression group change. In the docetaxel group one patient showed a relevant PD-L1 expression change (30 to 90%).

Within the subgroup of neoadjuvant chemoradiotherapy one showed a relevant increase in PD-L1 expression while the remaining patients showed no relevant change.

5.4.2 Influence of time on PD-L1 expression change between initial biopsy and surgery

In our study cohort the mean time from initial biopsy to surgery was 26 days. When analysing the influence of time to surgery on PD-L1 expression change we found no statistically relevant association between early and late relapse (p=0.95) as shown in figure 3.

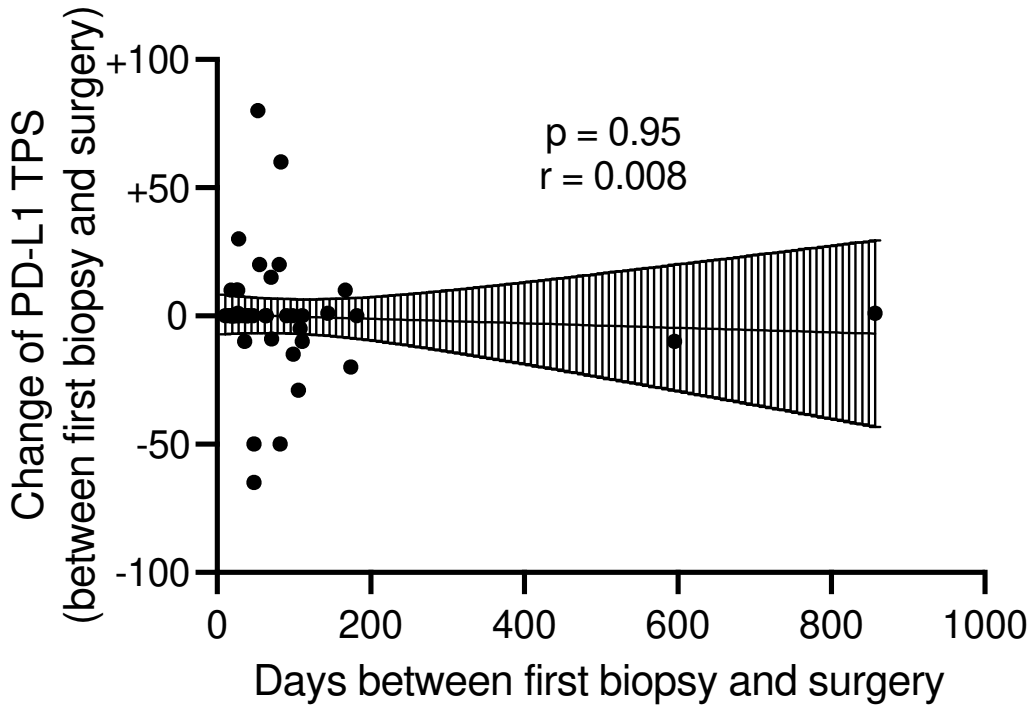

Figure 5 *Influence of time between initial biopsy and surgery on absolute PD-L1 expression change*

Table 4. Characteristics of surgical samples and matched preoperative biopsies							
	No Change of PD-L1 score group[1] (*N*=47)		**Change of PD-L1 score group (*N*=25)**		***P*-value[2]**	**Total (*N*=72)**	
	N	***%***	***N***	***%***		***N***	***%***
Gender							
Male	28	59.6	17	68.0	0.48	45	62.5
Female	19	40.4	8	32.0		27	37.5
Performance Status							
0	19	45.2	7	35.0	0.61	26	41.9
1	21	50.0	11	55.0		32	51.6
≥2	2	4.8	2	10.0		4	6.5
Smoking							
Never	4	10.5	1	5.9	0.58	5	9.1
Current or Former	34	89.5	16	94.1		50	90.9
Histology							
Adenocarcinoma	26	55.3	17	68.0	0.40	43	59.7
Squamous cell carcinoma	19	40.4	8	32.0		27	37.5
Other	2	4.3	0	0		2	2.8
Preoperative Tumor Stage							
1	7	15.2	4	16.7	0.98	11	15.7
2	18	39.1	9	37.5		27	38.6
3	21	45.7	11	45.8		32	45.7

Neoadjuvant Treatment							
No	38	80.9	18	72.0	0.39	56	77.8
Yes	9	19.1	7	28.0		16	22.2
Best response to neoadjuvant treatment							
Partial response	6	66.7	5	71.4	0.91	11	68.8
Stable disease	2	22.2	1	14.3		3	18.8
Progressive disease	1	11.1	1	14.3		2	12.5

[1]PD-L1 score group (PD-L1 negative, PD-L1 low (1-49%), PD-L1 high (50-100%))

[2]Chi[2] test[173]

5.5 PD-L1 expression change between surgical specimen and rebiopsy

In relapsed patients that underwent rebiopsy PD-L1 expression from rebiopsy could be compared to surgical specimen in 85 out of 87 cases. As previously mentioned, two patients were planned for surgery but underwent definitive chemoradiation instead. In these patients initial diagnostic biopsy PD-L1 expression was compared to rebiopsy PD-L1 assay. When categorized into clinically relevant PD-L1 expression groups (0%, 1-49%, and ≥50%), a relevant change was observed in 32 patients (36.8%). Specifically, 15 patients (17.2%) shifted to a higher PD-L1 expression group, 17 patients (19.5%) moved to a lower group, and 55 patients (63.2%) remained in the same group. Notably, there was a significantly smaller proportion of female patients among those who experienced a change in PD-L1 expression (table 3). Other baseline factors, such as performance status, smoking history, and tumor histology, showed no significant correlation with changes in PD-L1 expression (table 3).

5.5.1 Influence of adjuvant therapy

In total 29 patients (33.3%) received adjuvant therapy. Two patients (6.9%) received adjuvant chemoradiation, six (20.7%) radiation, and 21 (72.4%) adjuvant chemotherapy. However, adjuvant therapy was not significantly linked to changes in PD-L1 expression groups (p=0.53, table 3). Adjuvant chemotherapy regimen in our cohort consisted of a platinum backbone in combination with either pemetrexed (n=4), vinorelbine (n=12), gemcitabine (n=4) or etoposid (n=1). In the vinorelbine group PD-L1 expression changed only in 3 out of 12 cases. Two patients showed increased, while one showed PD-L1 expression group decrease. Those patients that were treated with pemetrexed (n=4) showed PD-L1 expression decrease group in one patient (100% to 0%) but not change in the remaining patients. Patients treated with gemcitabine (n=4) showed increase in one, decrease in one and no change in the remaining two patients respectively. The patient treated with etoposide formally showed PD-L1 expression group change. However, total PD-L1 expression change was only 1%. In total six patients received mediastinal radiation. Three of these patients showed PD-L1 expression decrease, while two showed no change and one increased.

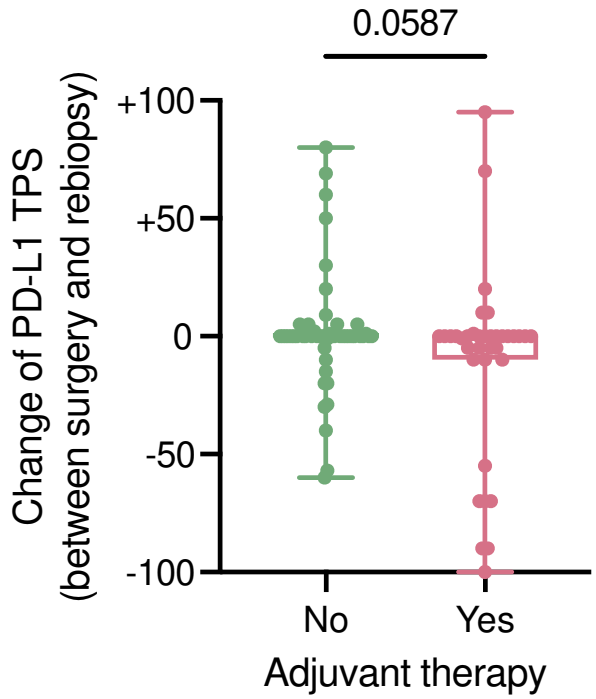

Figure 6 *Influence of adjuvant treatment on PD-L1 expression change between initial biopsy and surgical specimen*

Table 3. Characteristics of surgical samples and rebiopsies							
	No Change of PD-L1 score group[1] (*N*=55)		**Change of PD-L1 score group (*N*=32)**		***P*-value[2]**	**Total (*N*=87)**	
	N	***%***	***N***	***%***		***N***	***%***
Gender							
Male	28	50.9	24	75.0	0.03	52	59.8
Female	27	49.1	8	25.0		35	40.2
Performance Status							
0	22	44.9	10	40.0	0.67	32	43.2
1	23	46.9	14	56.0		37	50.0
≥2	4	8.2	1	4.0		5	6.8
Smoking							
Never	4	9.5	1	4.2	0.43	5	7.6
Current or Former	38	90.5	23	95.8		61	92.4
Histology							
Adenocarcinoma	37	67.3	16	50.0	0.28	53	60.9
Squamous cell carcinoma	17	30.9	15	46.9		32	36.8
Other	1	1.8	1	3.1		2	2.3
Postoperative Tumor Stage							
1	8	14.8	9	28.1	0.29	17	20.0
2	23	42.6	13	40.6		35	41.2
3	23	42.6	10	31.3		33	38.8

Adjuvant Treatment							
No	38	69.1	20	62.5	0.53	58	66.7
Yes	17	30.9	12	37.5		29	33.3
Time between surgery and rebiopsy							
<1 year	18	32.7	14	43.8	0.30	32	36.8
≥1 year	37	67.3	18	56.3		55	63.2

[1]PD-L1 score group (PD-L1 negative, PD-L1 low (1-49%), PD-L1 high (50-100%))

[2]Chi[2] test [173]

5.5.2 Influence of time on PD-L1 expression change between surgical specimen and rebiopsy

The median time from surgery to rebiopsy was 15 months, with 75% of the cohort having an interval of at least 9.5 months and 25% of the cohort an interval of at least 27 months between surgery and re-biopsy. When analyzing the influence of time to re-biopsy on PD-L1 expression change we found no statistically relevant association between early and late relapse (p=0.30, table 3).

5.6 Longitudinal analysis of PD-L1 expression in patients with relapsed NSCLC

In 72 patients (82.8%) PD-L1 expression could be compared between preoperative samples, matched surgical specimens and biopsy of relapsed disease. Thirty-nine patients (54.2%) showed at least one change into a different PD-L1 score group during the course of disease. Fourteen patients (19.4%) changed the PD-L1 score group twice, five (6.9%) of them being found in all different score groups. We could not observe a significant trend of change for PD-L1 expression during the course of disease. In the 39 patients with at least one change of score group, 11 (28.2%) patients had a constant increase in

PD-L1 expression whereas 12 (30.8) patients showed a constant decrease in PD-L1 expression. In 16 (41%) patients an increase of PD-L1 expression was followed by a decrease or vice versa (figure 2). When looking at the 33 patients who remained in the same PD-L1 score group for all three time points,19 (57.6%) subjects belonged to the PD-L1 negative group, six (18.2%) patients to the groups with a score of 1-49% and eight (24.2%) patients showed a constant score of ≥ 50%.

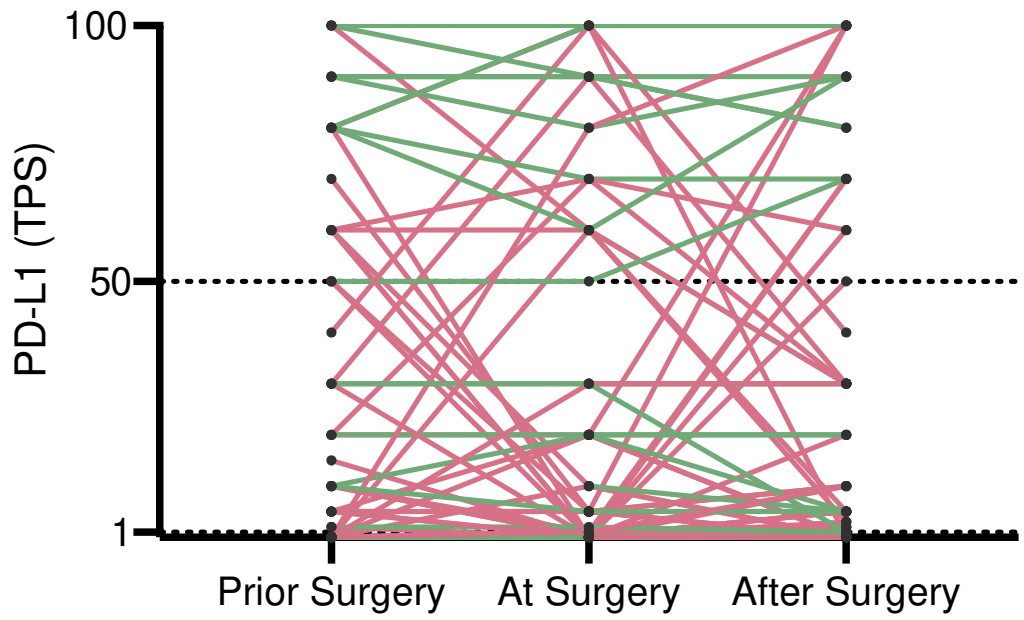

Figure 7 PD-L1 change of all patients that had PD-L1 expression measured prior to surgery, from surgical specimen and rebiopsy (N=72). Each line represents one case. Green lines represent cases that stayed within in the same PD-L1 score group, red lines represent cases in which PD-L1 expression changed at least once in the course of disease.[173]

5.7 Tumor heterogeneity

To investigate the possible influence of tumor heterogeneity on PD-L1 expression change, we re-assessed the surgical specimens of patients who changed PD-L1 expression score group every single time. This was the case in five of 72 patients. To answer this question, we evaluated the whole remaining surgical tumor block. In all patients between six to eight slides could be analysed for PD-L1 expression. We found that, on different slides, and often also in one slide, there were areas of different PD-L1 expression, sometimes ranging from 0 to 100% (Figure 8). If PD-L1 expression would have been assessed by more than one slide, using PD-L1 expression average might have lead to a more accurate representation of PD-L1 in NSCLC samples. In our cohort using PD-L1 expression average of all slides lead to PD-L1 expression scoring group change in two out of five patients.

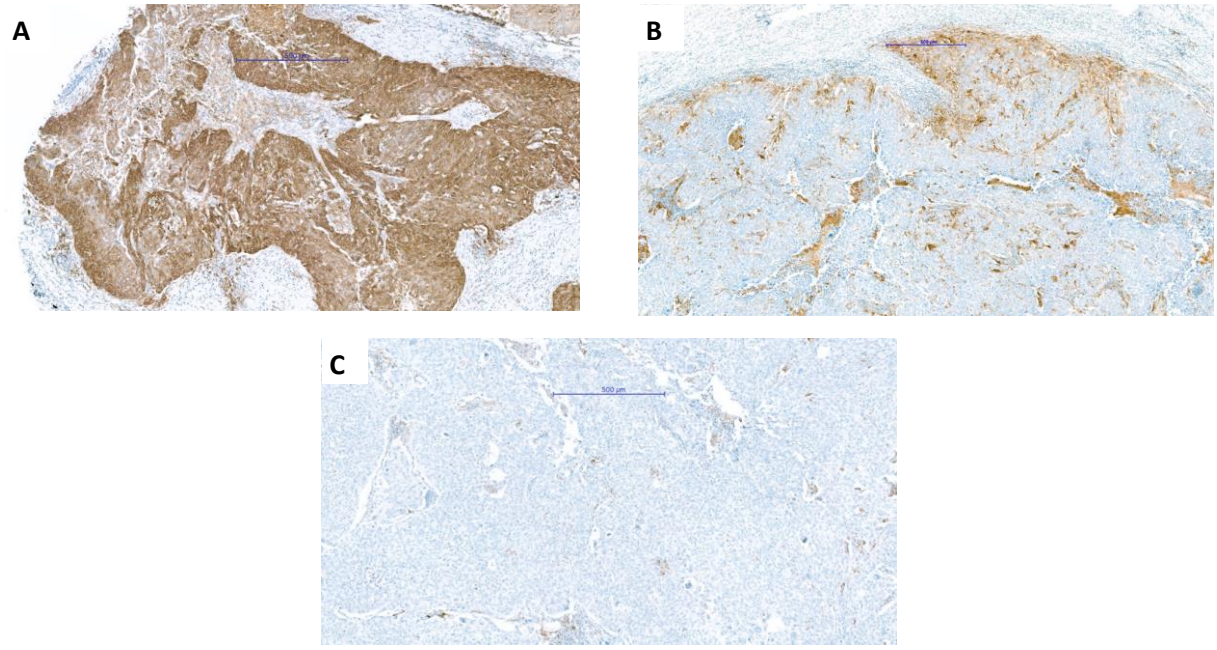

Figure 8 *Heterogeneity of PD-L1 expression in the same tumor: tumor proportion scores ranging from 100% (A), over 20 (B) to <1% (C).*[172]

6 DISCUSSION

This study focused on tracking PD-L1 expression over time in NSCLC patients with relapsed, metachronous disease. As far as we know, it is the first trial to examine preoperative biopsies alongside corresponding surgical samples from early-stage tumors, and subsequent re-biopsies from recurrent disease. Our results reveal that PD-L1 expression in NSCLC fluctuates significantly during the disease course in a substantial number of cases.

6.1 PD-L1 Expression a predictive biomarker in immune oncology

Programmed death-ligand 1 expression still is the most important predictive biomarker in non-small cell lung cancer (NSCLC) for the efficacy of immune checkpoint inhibitors (ICIs) targeting PD-L1 or PD-1. As by binding to PD-1 receptors on CD8 positive killer cells, PD-L1 can inactivate attacking immune cells and thereby suppress the immune response, allowing tumor cells to evade immune surveillance. Therefore, PD-L1 expression levels correlate with the ability of the tumor to evade the immune system, making it an ideal marker for therapies designed to counter this immune suppression. High PD-L1 expression, has been associated with increased overall survival and better response rates to ICIs in various clinical trials. Best known in lung cancer is the result of the KEYNOTE 024 study. In this phase III study 306 EGFR and ALK wildtype patients with stage IV NSCLC and PD-L1 of at least 50% were enrolled. Patients were 1:1 randomized and received either monotherapy with pembrolizumab or standard platin doublet chemotherapy. The overall response rate (ORR) was 44.8% for pembrolizumab compared to 27.8% for chemotherapy. The ICI led to remarkable increase in PFS (10.3 vs 6 months) and OS (30 vs 14 months).[174] A similar result was found for atezolizumab in the Impower110 study. In this phase III study 572 stage IV NSCLC patients with PD-L1 expression of at least 50% were enrolled. Patients received Atezolizumab or platinum-based chemotherapy. The mono immunotherapy led to a significant increase in PFS (8.2 vs 5 months) and OS. On the other hand several studies found that PD-L1 negative tumors show less response to ICI treatment.[120] One of the most prominent is the Keynote 042 study, which enrolled 1274 stage IV NSCLC patients with at least 1% PD-L1 expression. Participants were 1:1 randomized and received either platinum-based chemotherapy or pembrolizumab. The study found that PD-L1 high positive patients (PD-L1 ≥50%) benefited most from ICI, while those with PD-L1 expression of 1-49% had no statistically significant advantage when compared to chemotherapy (median OS 13.4 vs 12.1 months – HR 0.92). The median OS was even lower for patients up to 12 months of follow up.[120,175] Similar results were found by the Checkmate 026 study that examined Nivolumab vs platinum-based chemotherapy in PD-L1 positive NSCLC stage IV

patients.[176] In the neoadjuvant setting checkmate 816 assessed treatment with platinum-based chemotherapy in combination with nivolumab for 3 cycles before resection in stage IB (UICC 7th edition) - IIIA patients. The study found remarkable improvement in the event free survival (EFS) in all subgroups, even PD-L1 negative patients seemed to benefit, even if PD-L1 positive patients showed higher EFS.[80] In the adjuvant setting the Impower 010 study found improvement in disease free survival (DFS) in resected NSCLC patients only in PD-L1 positive patients. More recent analysis found a trend towards OS benefit which was driven by PD-L1 high positive patients (≥50%). These results led to approval for PD-L1 high positive patients by the EMA.

Based on these and other pivotal ICI approval studies PD-L1 expression is to date an important predictive biomarker for ICI treatment and an essential part of decision making in clinical oncology.

6.2 Limitations of PD-L1 Expression as immunological biomarker

Irrespective of its value however, PD-L1 expression alone lacks diagnostic accuracy for response to ICI treatment in NSCLC patients. Many publications found that a small but clinically relevant percentage of PD-L1 negative patients seems to benefit from checkpoint inhibitors. The Checkmate 017 and 057 study examined Nivolumab in metastasized NSCLC patients after progression in the first line setting for squamous and non-squamous cell carcinoma. In both studies PD-L1 negative patients were enrolled. Nivolumab was beneficial in all patients, but PD-L1 positive NSCLC patients did have higher ORR rates. In the PD-L1 negative subgroup ORR of 15% was reached, while the PD-L1 positive subgroup (≥1%) reached ORR of 20%.[117,177] Unfortunately high PD-L1 expression may also fail to predict response in a relevant number of cases. In the Keynote 024 study patients with PD-L1 over 50% and treated with pembrolizumab showed an ORR of only 45%. This implies that a large percentage of patients did not respond to ICI treatment irrespective of high PD-L1 expression status. When it comes to duration of response, patients with higher PD-L1 expression tend to have longer DOR. However, PD-L1 TPS cannot predict long time response to checkpoint inhibition in all patients. [123]

6.2.1 Influencing factors of PD-L1 expression and its predictive value

A possible explanation why some patients without PD-L1 expression benefit from checkpoint inhibitors may be the fact that PD-L1 expression is not static but may change over time [123,178–181]. Several studies found that PD-L1 expression levels may be induced by oncogenic driver activation [178,182], activation of NF-κB or release of IFN-γ by tumor infiltrating lymphocytes [183,184] and even oncologic treatment including chemotherapy and irradiation. [180,181] These finding were demonstrated across different tumor types including NSCLC. [184,185] Additional factors that may influence PD-L1

predictive value are study design and specific ICI, the tumor type and histological subtype of NSCLC, the PD-L1 assay [117,177,186,187] and also tumor heterogeneity. [125,188]

6.2.1.1 Oncogenic driver mutations

Several authors found associations of oncogene activation with induction of PD-L1 expression. Chen and colleagues examined the connection between EGFR and PD-L1 expression and found that EGFR mutated tumors are far more likely to express high levels of PD-L1. They showed that in fact activation EGFR may lead to PD-L1 upregulation irrespectively of other factors thereby confirming similar findings of previous publications. [177] In the early days of immune oncology it was therefore hypothesized that these tumors may respond to PD-1 / PD-L1 check point inhibitors. On the other hand, these tumors are often diagnosed in patients that are either light or never smokers, have low mutational burden and neoantigen production. This lack of neoantigen translates to low antineoplastic immunogenicity, which is associated with unfavourable response to checkpoint inhibitors. [132]

Several pivotal approval trials found that these patients do not respond to ICI treatment irrespective of PD-L1 expression level. In Checkmate 057, Keynote 010 and the POPLAR study, patients with oncogenic driver alterations were enrolled. In Checkmate 057 82 EGFR mutated patients were enrolled. In this subgroup of patients nivolumab was associated with a lower OS than docetaxel (10 vs 12.2 months). Similar results were found in the keynote 010 and POPLAR study.[177,189,190] As patients with oncogene-driven NSCLC were a small minority in these studies Mazieres and colleagues performed a large retrospective registry analysis to elaborate on this question. In total 551 patients from 24 centres in 10 countries were enrolled. Of these patients there were 271 KRAS, 125 EGFR, 43 BRAF V600E, 36 MET alteration, 29 HER2, 23 ALK ,16 RET and 7 ROS1 alteration. Unfortunately, PD-L1 status was only available in 214 of 551 patients. Around 33% of patients were PD-L1 negative, while 66% were positive. In the PD-L1 positive subgroup around one third showed a PD-L1 expression of 50% and above. Unsurprisingly, median PFS was low in most oncogenic dependent NSCLC. Overall, median PFS was 2.8 months. Median PFS was lowest for classical driver mutations like EGFR (2.1 months), ALK (2.5 months), RET (2.1 months), while smoke related molecular alterations like KRAS, BRAF and MET showed statistically significant higher PFS. Additionally, molecular altered NSCLC patients showed high rates of immune related adverse events associated with checkpoint inhibitor treatment. These results should further discourage the use of checkpoint inhibitors in NSCLC patients. In contrast to these findings Impower 150, a firstline phase III study for metastasized NSCLC patients enrolled oncogene driven NSCLC patients after exhaustion of all TKI treatment options including EGFR. Patients received carboplatin and paclitaxel in combination with atezolizumab and bevacizumab

(ABCP) compared to carboplatin and paclitaxel plus bevacizumab (BCP). In the overall study cohort PFS (8,3 vs 6,0) and OS (19,2 vs 14,7) benefit was demonstrated. This superior outcome was also found in the EGFR mutated subgroup. PFS for these patients was 10.3 months in the ABCP group compared to 6.9 months in the BCP group. These results led to the recommendation of ABCP for EGFR mutated NSCLC after progression exhaustion of TKI options by leading societies. However, doubts remain as the number of EGFR patients in the Impower 150 trial was small and as stated previously several other trials found contradictory results.[191] Today all approval studies for check point inhibitors exclude patients with classical oncogene dependent NSCLC like EGFR and ALK.

6.2.1.2 Oncological treatment

Another possible explanation for PD-L1 expression change might be oncological treatment. As previously stated, PD-L1 expression in tumor cells can be induced by release of IFN-γ by tumor infiltrating lymphocytes. As demonstrated by Lhuillier and several other authors tumor cell necrosis caused by oncologic treatment like irradiation leads to increased neoantigen presentation to immune cells and is associated with immune cell invasion and IFN-γ release.[192–194] Deng and colleagues found that irradiation increased PD-L1 expression and check point inhibitor efficacy in mice.[195] Based on these and similar results several authors investigated if neoadjuvant or adjuvant treatment in NSCLC patients is associated with PD-L1 expression change. Unfortunately to date there are contradictory results.

Zens and colleagues compared PD-L1 expression between initial biopsy and surgical tissue after neoadjuvant chemotherapy in 53 matched NSCLC samples. PD-L1 expression increased in 12, decreased in 7 and remained unchanged in 34 of 53 cases. These changes were not statistically significant.[180] In a similar study Choe and colleagues investigated the impact of neoadjuvant chemoradiotherapy on PD-L1 expression in stage III NSCLC. Their cohort consisted of 43 patients with localised NSCLC. 33 patients could be included in the final analysis. Of these 33 patients PD-L1 expression remained unchanged in 16 (48%), increased in 7 (21%) and decreased in 10 (30%). Concomitant radiochemotherapy therefore was not associated with statistically significant change in PD-L1 expression ($p = 0.887$).

In contrast to these studies, Rojkó and colleagues performed another neoadjuvant study also comparing PD-L1 before and after neoadjuvant Chemotherapy in NSCLC biopsy and surgical tissue samples. They found statistically significant decrease in PD-L1 expression after neoadjuvant chemotherapy with cisplatin and gemcitabine (p=0,051), while other chemotherapy combinations were not associated with significant PD-L1 expression change.[196]

In our study a total of 16 patients received neoadjuvant treatment. In 9 cases we found a PD-L1 expression score group change while 7 cases remained in the same group. Statistical analysis revealed no significant PD-L1 group change (p=0,392) Cisplatin and gemcitabine was used in 3 patients in our cohort. PD-L1 expression remained unchanged in all cases. All remaining chemotherapy regimen did not seem to have a significant impact on PD-L1 expression, although, due to the small group sizes, a statistical analysis seemed unreasonable.

In the adjuvant space Shu Yazaki and colleagues investigated the impact of chemotherapy on PD-L1 expression change between surgical specimen and rebiopsy from recurrence in a cohort of 83 matched breast cancer patients.[197] They found no statistically significant influence of chemotherapy on PD-L1 expression positivity between primary and metastatic tissue samples (p=0.23). Lacour and colleagues performed a similar study in NSCLC patients. They compared PD-L1 expression in 36 surgical tissue samples after resection and matched rebiopsy samples after recurrence. While they did find PD-L1 expression increase in 7 cases, adjuvant chemotherapy was not associated with statistically significant PD-L1 expression change.[198]

In our cohort 29 patients received adjuvant treatment. PD-L1 score group changed in 12 and stayed the same in 17 cases (p=0.53). We could neither demonstrate significant impact of adjuvant- chemo nor radiotherapy. However due to our smaller cohort size and heterogenous therapies these results cannot exclude a potential effect of adjuvant therapy on PD-L1 expression.

6.2.1.3 Study design and substances

Predictive value of PD-L1 expression for PD-1/PD-L1 checkpoint inhibitor treatment varies considerably between approval trials. In the Checkmate 026 study Nivolumab, an anti PD-1 antibody, did not show OS improvement in PD-L1 positive patients when compared to platinum-based chemotherapy. On the other hand pembrolizumab, also a PD-1 antibody, demonstrated PFS and OS benefit for a similar cohort in the Keynote 042 study.[104,176] As PD-L1 predicted response in Keynote 042 but failed in Checkmate 026 the most obvious explanation is a difference in drug efficacy.

Confronted with these findings one might suspect that Pembrolizumab shows higher anti-PD-1 affinity than Nivolumab. However, as checkmate 026 and keynote 042 differed in clinical endpoint (OS in keynote 042, PFS in Checkmate 816), study cohort (PD-L1 ≥5% in Checkmate 026, PD-L1 ≥1% in Keynote 042 overall higher percentage of PD-L1 high positive patients) and cross over a direct comparison between the trial results is not possible. Probably the most relevant difference in study design between the two trials was cross over. Keynote 042 did not allow for cross over to pembrolizumab

after progression under chemotherapy. In total only 20% of patients in the chemotherapy group received immunotherapy.[104] Checkmate 026 did allow crossover and 60% of patients in the control group received immunotherapy after progression, potentially reducing a potential OS benefit in the nivolumab group.[176]

Similar contradictions are found in the adjuvant setting. The IMpower010 study evaluated Atezolizumab as adjuvant therapy in patients with resected stage IB-IIIA NSCLC and PD-L1 expression of at least 1%. Patients received Atezolizumab or best supportive care following adjuvant chemotherapy. Atezolizumab improved DFS in PD-L1 positive patients (HR 0.66). However, only PD-L1 high positive patients showed clinically relevant benefit in DFS and OS. Therefore, Atezolizumab was only approved for PD-L1 high positive patients by the EMA. [199] In contrast the PEARLS study pembrolizumab increased DFS irrespectively of PD-L1 expression (HR=0.76) in a similar cohort and was therefore approved for all resected NSCLC patients after adjuvant chemotherapy. [82] Again study design and differences in patient cohort make a direct comparison and therefore inferences on the predictive value of PD-L1 expression impossible.

In summary, due to differences in trial design a direct comparison of checkpoint inhibitors efficacy trials is not possible. In absence of head-to-head trials however, the question of the difference in efficacy of these substances cannot be answered.

6.2.1.4 PD-L1 IHC antibodies

Apart from study design, the cross-study differences in the predictive value of PD-L1 expression might be explainable by the different PD-L1 assays. Unfortunately, Checkmate, Keynote, IMpower and Pacific all used different PD-L1 assays, each with distinct antibodies and scoring methods. The Keynote trials used the 22C3 assay, the Checkmate trials employed the 28-8 assay, the IMpower studies used the SP142 assay, and the Durvalumab trials used the SP263 assay. While the 22C3, 28-8, and SP263 assays tend to produce more comparable results, the SP142 assay, used in IMpower studies, typically detects fewer PD-L1-positive tumor cells but places greater emphasis on immune cells. These variations in assay sensitivity and focus could lead to differences in how PD-L1 expression is quantified across trials, potentially affecting eligibility thresholds and response rates to PD-L1 inhibitors, making direct comparisons between studies challenging.[200]

6.2.1.5 Histological subtypes and PD-L1 expression

The predictive significance of PD-L1 may also vary based on histologic subtype of NSCLC. This suggestion may be based on the comparison between the CheckMate 017 and CheckMate 057 study. Both evaluated nivolumab vs docetaxel in the second line after progression in metastasized NSCLC.

Checkmate 057 however enrolled nonsquamous cell carcinoma patients, while CheckMate 017 enrolled squamous cell carcinoma patients. Interestingly, both studies found contradicting results for value of PD-L1 expression. In the Checkmate 057 study patients with PD-L1 expression showed far better response to nivolumab. In the CheckMate 017 study however, this association could not be confirmed. As PD-L1 assays and study design are almost identical, differences in PD-L1 expression based on histology may be possible. Leading to the question if PD-L1 expression may be more predictive in adenocarcinoma of the lung.

6.2.1.6 Non recent biopsy and ICI treatment

In some PD-L1 negative NSCLC patients delay between diagnostic and treatment start might have enabled PD-L1 expression change. As previously stated, PD-L1 expression may change over the course of disease. As several factors may induce PD-L1 up- or downregulation in tumor cells time delay between initial biopsy and rebiopsy or omitting of rebiopsy at all may lead to inaccurate treatment decisions.

In this context it is worth mentioning that PD-L1 assessment requirements varied widely between approval trials. In the Checkmate studies, either recent or archived tumor samples collected within 3 to 6 months before patient enrolment were necessary. [143,171,193] The Keynote trials, required tumor samples from the initial diagnosis of metastatic disease untreated by prior therapies. However, no time limit between initial diagnosis and enrolment was provided. [82,105,189,192]. PACIFIC only mandated archived tumor tissue for PD-L1 evaluation. A time limit or recent biopsy was not required.[194] The same is true for the IMpower trials. [195,196]. In conclusion, only the checkmate studies mandated a fixed time limit between initial diagnosis and treatment start. However, even the given time window of 3 to 6 months seems may be insufficient. Recent biopsy for PD-L1 assessment was never required for enrolment. Therefore, time from biopsy to treatment start may vary considerably. Longer time periods between initial biopsy and treatment start may lead to clinically relevant PD-L1 change.

In our study we examined the PD-L1 expression change between initial biopsy, surgical specimen and rebiopsy at recurrence. PD-L1 expression in matched cases at the various time points varied considerably. When we compared PD-L1 expression in initial biopsy and surgical tissue, we found treatment relevant PD-L1 expression change in 32 of 87 cases. When evaluating these patients for adjuvant ICI treatment following IMpower 010 protocol without recent PD-L1 expression testing from surgical tissue 5 of 87 patients would not qualify for adjuvant check point inhibitor treatment although indicated. On the other hand, another 5 of 87 patients would receive check point inhibitor treatment based on IMpower 010 and will not benefit from this treatment. Therefore 10 of 87 patients would have

received the wrong treatment recommendation. We conclude that PD-L1 retesting from surgical tumor specimen is necessary in all cases in order to accurately guide treatment decisions.

The same holds true for relapsed disease. In our real-life cohort patients with relapsed disease received rebiopsy in only 87 of 136 cases. Omitting of rebiopsy may be reasonable in some cases, as relapse location may be difficult to reach, or interventional risk may be disproportionately high. However, in our study rebiopsy lead to a clinically relevant PD-L1 expression group change in 36,8% of patients when compared to surgical tissue. In eight patients high PD-L1 expression (≥ 50%) decreased into the PD-L1 low (1-49%) or negative (0%) group, changing the recommended treatment as patients with low or PD-L1 expression usually receive chemotherapy in addition to ICI treatment.[107,109,201] In these cases, omitting of retesting would have led to mono ICI treatment in PD-L1 low or negative patients, which was associated with reduced PFS and OS in most approval studies and would have potentially impaired these patients quality of life and prognosis.

6.2.1.7 Tumor heterogeneity

Aside from alterations in PD-L1 expression, discrepancies in measured PD-L1 levels may also arise from misrepresentation caused by tumor heterogeneity.[188] The heterogeneous nature of tumors often described as tumor mosaic is caused by tumor evolution and can lead to focal higher or lower PD-L1 expression within tumors.[188,202,203] Casadevall and colleagues analysed PD-L1 expression in different regions of 144 surgical NSCLC samples. Of these 144 cases 50 were squamous cell carcinoma and 94 were adenocarcinoma. The authors compared two distinct tumor areas of each surgical sample. PD-L1 expression discordance was found in 10% of adenocarcinoma and 19% of squamous cell carcinoma.[202]

In our study, we found clinically relevant PD-L1 expression score group change in a significant number of patients. In order to assess possible influence of tumor heterogeneity we reassessed surgical specimens of five patients who changed score group every single time histology for PD-L1 testing was gained. In these cases, all available surgical tumor blocks underwent PD-L1 staining. As suspected, we found areas of different PD-L1 expression in all surgical tumor samples, however clinically relevant PD-L1 expression change was only found in two of five patients. Still, this implies a clinically relevant PD-L1 expression misrepresentation in 40% of patients and therefore might have potential implications for neoadjuvant, adjuvant and palliative treatment decisions. In the neoadjuvant setting, PD-L1 expression is performed in tissue biopsy samples, which are often small and may not represent the whole tumor. If biopsy samples are wrongly classified as PD-L1 negative, patients may not receive neoadjuvant immunochemotherapy, as checkmate 816 is only approved for PD-L1 positive patients. The same is true in the adjuvant setting. As currently only one slide of resected NSCLC tumors is used for PD-L1

assessment, PD-L1 misrepresentation is very likely. In case of relapse rebiopsy is often performed using needle aspiration and therefore gaining only minimal amounts of tissue. Again, leading to a high risk of PD-L1 misclassification and potentially impacting prognosis.

Differences of PD-L1 expression between biopsy samples and surgical specimen were analysed in several previous studies. Ilie and colleagues assessed 160 patients with operable NSCLC using whole surgical tissue sections and compared them to corresponding lung biopsies. They observed a high discordance rate of 48%. In all cases with PD-L1 expression difference between biopsy and surgical specimen, biopsies underestimated PD-L1 However Discordance rate of biopsies could be decreased when at least six biopsy samples were taken and used for PD-L1 immunohistochemistry analysis. [204]

This heterogeneity within a primary tumor might also be the reason, why PD-L1 expression varies between primary tumor and locally or distant metastases as described by several publications in recent years. [125,203,205] If cells with high PD-L1 expression in a tumor with predominantly PD-L1 negative cells, form metastases rebiopsy of these metastases may find high PD-L1 expression, while PD-L1 expression in the primary may be low. This potential difference in PD-L1 expression between primary and metastases may also explain why in some patient's metastases respond to treatment, while the primary remains unaffected.

In summary tumor heterogeneity potentially plays an important role in PD-L1 expression assessment. As PD-L1 assessment is currently not standardized and only one slide or biopsy core is used for PD-L1 IHC, approval of potentially lifesaving checkpoint inhibitors should not be based on PD-L1 expression alone. Furthermore, PD-L1 assessment must be standardized and at least two slides of surgical tissue and six cores from biopsy should be recommended.

6.3 Limitations of this study

The main limitation of this study is its retrospective nature and the relative low number of matched PD-L1 samples. For our analysis we screened over 600 resected NSCLC patients. However, rebiopsy was only performed in 87 patients. Furthermore, PD-L1 expression was initially only re-performed in 70 of these patients. In 17 cases the PD-L1 assay had to be reperformed for our study, as the case managers did not request PD-L1 expression from rebiopsy at the time of rebiopsy. In some cases, biopsy samples were collected in external centres and PD-L1 restraining could not be performed. All these factors reduced the number of cases that could be compared. Still our cohort is larger than most other studies in this field. When the influence of chemotherapy or radiation on PD-L1 expression is considered, the relatively low number of patients in our cohort reduces the significance of our findings. The same is true

for our analysis of tumor heterogeneity, as we only performed additional PD-L1 assays in five of 87 cases. Furthermore, preselection of thesefive cases based on most pronounced PD-L1 expression change means that the high tumor heterogeneity found, may not be transferable to the whole cohort of patients. Influence of tumor heterogeneity, therefore, may be lower as suspected. Still, we believe our findings to be relevant for clinical practise. However, further research especially prospective studies are needed to answer the question of significance of our results.

Furthermore, molecular testing is not homogeneous in our cohort, as patients were enrolled between 2015 and 2019. In this time frame next genome sequencing panels did change in our but also in external centres in Styria. After 2019 the Ion AmpliSeq Colon/Lung Cancer Panel V2 and Archer Fusion Plex Expanded Lung Panel were used in all cases. Still, in 59 cases testing for RET and NTRK is missing. However, due to the scarcity of RET and NTRK inclusion of a relevant number of patients, that harbour these mutations, in our study cohort seems unlikely.

6.4 Conclusions

In summary, our results show that PD-L1 expression does change of the course of disease in NSCLC patients. The most likely cause of PD-L1 change in our view is tumor heterogeneity next to tumor evolution and transcriptional changes. In contrast to previous studies, we did not find clinical significant impact of oncologic treatment on PD-L1 expression. Based on our findings, we suggest the formation of consensus guidelines for the assessment of PD-L1 testing, detailing recommended amount of tissue in biopsy for PD-L1 staining and number of slides from surgical specimen to minimize influence of tumor heterogeneity. In the meantime, treatment with ICIs should not be based on specific PD-L1 expression levels.

7 References

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