Thesis

Routine Laboratory Parameters as Biomarkers of Response to Immune Checkpoint Inhibitors in Lung Cancer

submitted by

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Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Graz, 07.08.2024 *Johanna Koinig-Trevino m.p.*

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Zusammenfassung

Die Einführung von Immun-Checkpoint-Inhibitoren hat die Behandlung von Patient*innen mit Lungenkarzinomen grundlegend verändert und die mittlere Überlebenszeiten für Responder deutlich erhöht. Allerdings sprechen nur rund ein Drittel der Patient*innen auf diese Therapie an, und einige Patient*innen erleiden lebensgefährliche autoimmun-mediierte Nebenwirkungen. Gute prädiktive Biomarker sind kaum vorhanden, und die beiden derzeit etablierten Marker, PD-L1 Expression und Tumour Mutational Burden, müssen beide anhand von Gewebeproben untersucht werden. Das Ziel dieser Arbeit war die Evaluierung von Parametern des „großen Blutbilds“ zur Vorhersage von Ansprechen auf die Therapie mit Immun-Checkpoint-Inhibitoren, um neue Hypothesen zu generieren. Die Blutproben wurden vor Behandlungsbeginn und drei Monate später entnommen. Erhöhte CRP-Werten vor Behandlungsbeginn und drei Monate nach Behandlungsbeginn waren in der Kaplan-Meier Analyse und Cox Regression mit kürzerem Gesamtüberleben assoziiert. CRP ist ein vielversprechender, kostengünstiger, und nicht-invasiver Biomarker, und sollte in weiteren prospektiven Studien untersucht werden.

Abstract

The introduction of immune checkpoint inhibitors revolutionised the treatment of lung cancer and greatly increased median survival. However, only about one third of patients respond to immune checkpoint inhibitor therapy and some patients suffer from life-threatening autoimmune-mediated adverse events. Predictive biomarkers are very limited, and the two established markers – PD-L1 expression and tumour mutational burden – are both tissue-based. The aim of this thesis was to evaluate whether a set of routine laboratory parameters could predict response to immune checkpoint inhibitors in lung cancer. Blood samples were obtained at baseline and three months after the initiation of treatment from NSCLC patients treated with anti-PD-1/PD-L1 therapy. Elevated pre-treatment CRP and CRP at the three-month mark were associated with shorter overall survival in Kaplan-Meier analysis and Cox regression analysis. If confirmed by further prospective studies, CRP could be a low-cost, and non-invasive biomarker of response to ICIs.

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List of abbreviations

ADL	Activities of daily living
AE	Adverse event
AEC	Absolute eosinophil count
ALK	Anaplastic lymphoma kinase
ANC	Absolute neutrophil count
APC	Antigen presenting cells
AUC	Area under the curve
CI	Confidence interval
COX	Cyclooxygenase
CRP	C-reactive protein
CT	Computer tomography
dNLR	Derived neutrophil-to-lymphocyte ratio
EBUS-TBNA	Endobronchial ultrasound-guided transbronchial needle aspiration
EGFR	Epidermal Growth Factor Receptor
ESMO	European Society for Medical Oncology
EU	European Union
EUS-FNA	Endoscopic ultrasound-guided fine needle aspiration
FDA	Food and Drug Administration
FDG-PET	Fluorodeoxyglucose positron emission tomography
HER2	Human epidermal growth factor receptor 2
HLA	Human leukocyte antigen
HR	Hazard ratio
IARC	International Agency for the Research of Cancer
ICI	Immune checkpoint inhibitor
IFNγ	Interferon gamma
IHC	Immunohistochemistry
IL	Interleukin
KRAS	Kirsten rat sarcoma viral oncogene homologue
MHC	Major histocompatibility complex

MRI	Magnetic resonance imaging
NGS	Next-generation sequencing
NK cells	Natural killer cells
NLR	Neutrophil-to-lymphocyte ratio
NSCLC	Non-small cell lung cancer
ORR	Objective response rate
OS	Overall survival
PCR	Polymerase-chain reaction
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
PFS	Progression-free survival
RBC	Red blood cell count
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SCLC	Small cell lung cancer
SIADH	Syndrome of Inappropriate Antidiuretic Hormone Production
TCR	T-cell receptor
TPS	Tumour proportion score
WBC	White blood cell count
95% CI	95% confidence interval

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1 Introduction

1.1 Lung cancer

1.1.1 Epidemiology

In 2019, lung cancer was the second most common new tumour diagnosis in Austria for men and women. There was a total of 4831 new cases, with 2770 new cases in men and 2061 new cases in women. Compared to other cancers, lung cancer was responsible for the highest number of cancer deaths in men (21%) and the second highest number in women (17%) in Austria. For women in Austria, the incidence of lung cancer as well as lung cancer mortality has been increasing dramatically in the last decades. (1)

Globally, lung cancer was the leading cause of cancer deaths in 2020, causing 1.8 million deaths or 18.4% of all cancer deaths, according to the World Health Organisation. It was also the second most diagnosed cancer after female breast cancer, with 2.2 million new diagnoses or 11.4% of all new cases. It is the most commonly diagnosed cancer in men with 14.3% of new cases worldwide, followed by prostate cancer (14.1%), and the third most commonly diagnosed cancer in women with 8.4% of new cases, after breast cancer (24.5%) and colorectal cancer (9.4%).

The incidence rates of lung cancer are significantly higher in high-income countries compared to low-income countries. However, this trend is expected to shift as the majority (80%) of smokers over the age of 15 now reside in low-income and middle-income countries. Among men, the regions with the highest incidence rates of lung cancer include Micronesia/Polynesia, Eastern and Southern Europe, Eastern Asia, and Western Asia, with Turkey having the highest rate globally. In contrast, lung cancer incidence rates in Africa remain relatively low. Among women, the regions with the highest lung cancer incidence rates include Northern America, Northern and Western Europe, Micronesia/Polynesia, and Australia/New Zealand, with Hungary having the highest rate globally.(2, 3)

In many high-income countries, the incidence of lung cancer is declining in men due to declining tobacco use. This is not yet the case for women in most countries, due to the historically later uptake of tobacco use in women. (4)

1.1.2 Classification

The two main types of lung cancer are non-small cell lung cancer (NSCLC, 85% of all diagnoses) and small-cell lung cancer (SCLC, 15% of all diagnoses). NSCLC is a heterogenous group, in which adenocarcinomas form the most common subtype, followed by squamous-cell carcinoma. (5) Further subtypes include large cell carcinoma, and less frequently, adenosquamous carcinoma, sarcomatoid carcinomas, and others. Classification depends on morphology, immunohistochemistry, and molecular testing including genetic testing. (6) The most common targetable genetic alterations in adenocarcinomas are EGFR- and KRAS- activating mutations followed by ALK and ROS1 rearrangements, BRAF mutations, MET exon 14 skipping mutations and MET amplifications, RET gene fusions and HER2 mutations. EGFR- and HER2-mutations and ALK rearrangements are more common in non-smokers, whereas KRAS- and BRAF-mutations are more common in smokers. (7)

1.1.3 Risk factors

1.1.3.1 Smoking

The main established risk factor for the development of lung cancer is smoking. The IARC released a Monograph on tobacco and secondhand smoke in 2004, which highlighted significant discoveries regarding the carcinogenic risk associated with tobacco. One crucial finding was that the likelihood of developing lung cancer from smoking depends most strongly on the length of exposure, as well as the overall dose and the age at which individuals begin smoking. The study also definitively established that smoking increases the risk for all histological types of lung cancer, and that the carcinogenic effects of smoking are affecting both men and women similarly. Furthermore, it concluded that quitting smoking reduces the risk, with early cessation yielding the greatest benefits. (8)

A study conducted by Simonato et al. in Europe revealed that men who were actively smoking had a 24-fold higher risk of developing lung cancer compared to lifelong non-smokers, and that women who were active smokers had an 8.7-fold increase in risk compared to lifelong female non-smokers. Male ex-smokers still had a 7.5-fold higher risk, and female ex-smokers had 2-fold higher risk of developing lung cancer. (9)

In the European Union, about 85% of lung cancer deaths can be attributed to smoking cigarettes. (10, 11) Considering all this, it is concerning that in 2019, 18.4% of the EU population aged 15 and over smoked cigarettes daily. (12)
Smoking cigars, cigarillos and pipes also has been established to have a similar carcinogenic effect as smoking cigarettes. (13)

1.1.3.2 Secondhand smoke

The 2004 IARC Monograph also established secondhand smoke as a carcinogenic agent which increases the risk of lung cancer. Smoking cigarettes always comes with secondhand smoke which consists of exhaled mainstream smoke and side stream smoke, and the size of the particles in secondhand smoke is generally smaller than in mainstream smoke. Smoking areas have a three times higher amount of respirable suspended particles compared to non-smoking areas. Besides respirable suspended particles, secondhand smoke contains a range of other agents, like carbon monoxide, nicotine, nitric oxid, carbonyl compounds and others. (8)

Several studies have investigated the relationship between spousal exposure to secondhand smoke and lung cancer, and the IARC's meta-analysis found an increased risk in developing lung cancer if an individual's spouse is a smoker. For women, the risk increased by 24%, and for men, by 37%. The data also shows a dose-response relationship, with the risk increasing with increased exposure. (8)

In the IARC's meta-analysis, similar data was obtained for workplace exposure to secondhand smoke, with a pooled increased risk for women of 20%, and for men of 12%, although the results were not statistically significant, probably due to small sample size. (8) Another meta-analysis performed by Stayner et al. in 2007, which included 22 studies, showed a 24% increased lung cancer risk in nonsmokers exposed to environmental tobacco smoke in the workplace, and a two-fold increase in nonsmokers who reported high exposure. (14)

1.1.3.3 Radon

Radon is a naturally occurring radioactive gas and is responsible for most of the natural exposure to ionizing radiation. There is local variation in the amount of naturally occurring radon, and certain workers are more exposed, especially those working in mining. (10) A European meta-analysis of 13 case-control studies on radon exposure in homes showed a

linear dose-response relation and an increase in relative risk of 16% per 100 Bq/m3 in corrected radon exposure. It estimates that residential radon exposure causes 9% of deaths from lung cancer in Europe. (15)

1.1.3.4 Outdoor air pollutants

Outdoor air pollution is a complex mixture of pollutants stemming from transport, industrial activity, burning of biomass, domestic sources, and anthropogenic sources. The IARC has categorized air pollution and particular matter from outdoor air pollution as carcinogenic to humans. The IARC estimates that air pollution causes 223,000 lung cancer deaths annually worldwide. (16)

1.1.3.5 Occupational exposure to carcinogens

The IARC has recognized a long list of carcinogens in relation to lung cancer, including asbestos, arsenic, polycyclic aromatic hydrocarbons, coal, diesel engine exhaust, silica, coal, iron and steel founding, nickel compounds, soot, rubber manufacturing, welding fumes, and others. (17)

1.1.4 Clinical presentation

Most patients already present with symptoms at the time of diagnosis. According to a systematic review by Spiro et al. from 2007, around 90% of patients have symptoms at the time of diagnosis. About a third of the symptoms are a result of the primary tumour, a third are systematic in nature, like anorexia or fatigue, and a third are related to distant metastases. The most common symptom was cough, followed by weight loss, dyspnea, and chest pain. Patients who are asymptomatic at time of diagnosis have a better 5-year survival rate compared to those who are symptomatic, with those who have symptoms related to metastases having the worst survival rates. (10, 18)

Tumours that are located toward the center of the lung are more likely to cause symptoms than tumours located in the lungs' periphery. The most common symptoms caused by the primary tumour are cough, dyspnea, chest pain, and hemoptysis.
Intrathoracic spread of the tumour can cause a range of symptoms depending on the infiltrated structure. These might include nerves, like the recurrent laryngeal nerve or the phrenic nerve, vascular structures, the heart, the esophagus, or the chest wall and pleura. Invasion of the pleura and chest wall causes localized chest pain, which is a symptom that affects up to 50% of patients over the course of the disease. (10, 18)
Lung cancer most commonly spreads to the bones, liver, adrenal glands and intraabdominal lymph nodes, brain and spinal cord, and lymph nodes and skin. Bone pain from metastases is present in about 25% of patients at presentation. (18)

Patients with lung cancer can also develop paraneoplastic syndromes. It is estimated that this affects 10% of patients with bronchogenic tumours. Paraneoplastic syndromes are not a result of the physical effects of the primary tumour or metastases but are due to the production of biologically active substances like hormones or cytokines or due to an immune response and the formation of antibodies. Paraneoplastic syndromes in lung cancer patients are often endocrine syndromes like hypercalcemia, SIADH production, or Cushing syndrome, but can also involve neurologic syndromes like Lambert-Eaton syndrome, skeletal syndromes, metabolic syndromes, and others. Presence and severity of paraneoplastic syndromes do not correlate with tumour size, actually, sometimes they present before the primary malignancy has been diagnosed. (18)

1.1.5 Diagnosis and staging

According to the 2023 interdisciplinary guideline of the German Respiratory Society and the German Cancer Society on the prevention, diagnosis, treatment, and follow-up of lung cancer, after initial suspicion due to symptoms or a positive chest radiograph, basic diagnostics should be carried out if there is any clinical suspicion for lung cancer. The patient's history should be investigated, especially regarding potential risk factors such as smoking and occupational exposure to carcinogens. A basic lab workup should be obtained – including differential hematology, coagulation tests, liver enzymes and renal function parameters. Furthermore, a chest radiograph should be obtained as a first means of imaging. A CT of the chest and upper abdominal region can be obtained to investigate a suspicious chest radiograph and is the most sensitive imaging technique for the detection of lung cancer. Furthermore, a bronchoscopy can be carried out. Also, the ECOG/WHO Performance Status should be evaluated. (10)

For staging and risk assessment, both the ESMO Clinical Practice guidelines, the British National Institute of Health and Care Excellence guideline as well as the German Cancer Society's guidelines recommend carrying out a contrast enhanced staging-CT scan of chest and upper abdomen, including the liver, kidneys, and adrenal glands. If available, an MRI of the central nervous system is recommended for all patients with known or suspected lung cancer, although recommendations on this vary internationally and some guidelines recommending this only for patients with curative treatment intent or patients with neurological symptoms. An MRI may also be useful in Pancoast tumours and tumours which infiltrate the mediastinum, although the German guideline only recommends obtaining one in case of curative intent. An FDG-PET/CT can be useful in staging for the detection of distant metastases and the assessment of metastases in mediastinal lymph nodes, because it can differentiate between atelectasis and tumour. It is also useful in finding further pulmonary tumours, and it can help with the differentiation between a benign pulmonary nodule and a malign tumour. In patients with SCLC, an FDG-PET/CT scan should be obtained for all patients without known distant metastases. If bone metastasis is clinically suspected, either a bone scintigraphy or an FDG-PET-CT is recommended.
(7, 10, 19)

A pathological confirmation of the diagnosis as well as molecular testing is often necessary. The recommended method of obtaining a sample would be a bronchoscopy with sampling of the tumour, for example via endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) or endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA). In patients with curative intent, a biopsy of any suspicious intrathoracic nodes over 10mm size should be obtained via EBUS-TBNA or EUS-FNA.
Cytology from bronchoscopy can be accepted for treatment management if all molecular testing can be performed. The least invasive way of getting a cytology is via sputum cytology, which is an option if the tumour is centrally located and the patient has a high morbidity. For peripheral tumours, other options would be percutaneous image-guided needle aspiration or biopsy of the tumour. Sometimes adequate tissue sampling is necessary for individual treatment decision. More invasive procedures like a surgical biopsy should only be used if a less invasive approach did not bring conclusive results or was not possible. (7, 10, 19)

Staging and diagnosis of lung cancer should always include the TNM-classification of the tumour. The clinical TNM-classification (cTNM) can aid in choosing the right treatment. T describes the primary tumour and its size and infiltration of adjacent tissues on imaging, N describes the location of the involved regional lymph nodes, and M the presence, number, and location of distant metastases. Pathological classification can be obtained postoperatively, as the resection of the primary tumour (pT), resection of regional lymph nodes (pN) or biopsies of metastases (pM) are required. (10, 20)
The eight edition of the lung cancer stage classification was introduced in 2017. Tumour stages are defined according to the TNM-classification and range from 0 to IV. These stages are relevant for prognosis and treatment. (20)

After the diagnosis has been made and the tumour has been histologically classified, molecular testing can be performed on the available tissue or cells.
For stage IV NSCLC patients EGFR- and BRAF V600 as well as ALK-, ROS-1-, RET- and NTRK1-3-translocations are relevant for choosing a suitable first-line therapy. For stage IV and some stage III patients, tumour cell PD-L1 expression rate should be immunohistochemically assessed. The result is the percentage of tumour cells expressing PD-L1 on their surface (tumour proportion score). (10)

1.1.6 Therapeutic options

Therapy will greatly depend on the stage of lung cancer at the time of diagnosis, as well as the patient's comorbidities, age, and symptoms.
Typical treatment options include surgical treatment with resection of the tumour, radiation therapy, radio chemotherapy, immunotherapy, chemotherapy, and targeted therapies. These options can be combined or used as a stand-alone treatment. (10)

For stage I and II patients who are operable, the goal should be curative surgical resection of the tumour as well as dissection of the lymph nodes. For tumours >2cm the entire lobe of the lung should be removed, for tumours <2cm only the lung segment. (10)
Video-assisted thoracic surgery (VATS) is the preferable method with less adverse events and less postoperative pain in comparison to conventional open thoracic surgery. (21)
The German guidelines do not recommend neoadjuvant chemotherapy for stage I patients. For stage II patients, they recommend discussing a preoperative systemic therapy based on factors like PD-1 expression, risk for incomplete resection, comorbidities, patient compliance and patient preference. (10) After a full resection of the tumour, the German guidelines suggest offering stage II patients in good health adjuvant chemotherapy, preferably a combination chemotherapy containing cisplatin over four cycles of treatment. Vinorelbine is the most established combination, other common combinations are with docetaxel, gemcitabine or pemetrexed. (10)

Stage II patients with an EGFR-mutation can be treated with the tyrosine-kinase-inhibitor Osimertinib for three years after resection and adjuvant chemotherapy. An ADAURA double-blind, phase 3 trial showed that 90% of stage II-IIIA patients with an EGFR-mutation in the adjuvant Osimertinib group versus 44% of those in the placebo group were alive and disease-free after 24 months. (22)
The immune checkpoint inhibitor Atezolizumab can be offered to patients with a PD-L1 expression ≥50% of tumour cells after R0-resection and adjuvant chemotherapy. (10) The Impower010 phase 3 trial showed that adjuvant atezolizumab administered over the course of one year improved disease-free survival in comparison to best supportive care in stage II-IIIA patients with a PD-L1 expression of 1% or more of tumour cells. The benefit was most pronounced in patients with an expression of PD-L1 on 50% or more, the unstratified HR for this group was 0.43 (95% CI 0.27-0.68). (23)

About 12-25% of patients diagnosed with an early stage of lung cancer are inoperable due to comorbidities. As an alternative to surgery stereotactic radiotherapy can be offered, although studies suggest sublobar resection has a more favorable outcome in terms of overall survival. (10, 24)
Stereotactic body radiotherapy uses a method of external beam radiotherapy that makes it possible to accurately apply a high dose of radiation to a small target volume whilst protecting the surrounding tissue. Doses are administered in fewer fractions than in conventional radiotherapy. (25)

Patients with stage III lung cancer are heterogenous group and need to be treated according to their specific subgroup. Depending on the subgroup and molecular markers, different multimodal treatment options are available. Resectable patients can be either treated with surgery followed by a cisplatin-based adjuvant chemotherapy, which can be followed by adjuvant immunotherapy with atezolizumab or adjuvant treatment with the tyrosine-kinase inhibitor Osimertinib. Neoadjuvant chemotherapy with cisplatin and taxane followed by resection can be an option for some patients. (10)

ICIs are now part of the first-line treatment of unresectable stage III patients, either as monotherapy, immunochemotherapy, or after chemoradiotherapy. (26)
The addition of durvalumab to radiochemotherapy was one of the significant therapeutic advances in the treatment of stage III patients, as it not only greatly improved median survival but also four-year survival in the PACIFIC trial. (27) In a subgroup analysis, the benefits were shown for patients with a tumour cell PD-L1 expression of ≥1%. (28)
Patients who are unfit for either surgery or radiochemotherapy and who have a PD-L1 expression >50% can be offered monotherapy with cemiplimab. The EMPOWER-Lung 1 study showed improved overall survival and progression-free survival in patients with advanced NSCLC with a PD-L1 expression >50% treated with cemiplimab monotherapy compared with platinum-doublet chemotherapy.(29)
Immune checkpoint inhibitors are also being trailed (?) as a neoadjuvant treatment in resectable stage III patients. (30)

Stage IV patients have a poor prognosis, with 5-year survival rate estimated at around 6%. (31) Treatment can improve survival, especially if targeted treatment is possible. (32)

The standard treatment used to be a platinum doublet chemotherapy, however this has drastically changed with the introduction of immunotherapy and targeted therapies. (26) Before treatment, it should be assessed if the disease is potentially oligometastatic and can be treated with a curative intent. (10) Apart from this situation, treatment of stage IV patients generally follows a palliative intent, and patients should be treated systemically. (26)
Tumour tissue of all stage IV patients should be molecularly tested for EGFR-mutations, BRAF V600 mutation, ALK-fusions, RET-fusions, and NTRK1-3 fusions, as there are very successful targeted treatments in the form of tyrosine kinase inhibitors and monoclonal antibodies available for these mutations. (10)

ICIs play a very important role in the treatment of stage IV patients without targetable mutations. Patients with no targetable mutation but a PD-L1 expression of greater 50% of tumour cells or greater 10% of immune cells can be treated with atezolizumab, cemiplimab or pembrolizumab monotherapy or with pembrolizumab or nivolumab in combination with ipilimumab and chemotherapy as a first line therapy. (10)
The KEYNOTE-24 study showed significantly longer progression free and overall survival in patients with advanced NSCLC treated with pembrolizumab monotherapy compared with chemotherapy. The survival after 6 months was 80.2% in the pembrolizumab group compared with 72.4% in the chemotherapy group. There were fewer adverse events in the Pembrolizumab group (73.4% versus 90.0%) (33)

The introduction of the combination platinum doublet chemotherapy with pembrolizumab significantly improved the treatment of stage IV patients, as it improved objective response rate, progression-free and overall survival in both squamous and non-squamous histology, irrespective of PD-L1 expression. (26, 34, 35) The combination increased immune-related adverse events, however, not grade 3 or 4 AEs. (26, 35) Irrespective of PD-L1 expression, patients without targetable mutations can now be offered a variety of chemoimmunotherapy combinations including a variety of ICIs, which show similar improvement of survival as pembrolizumab. (10)

Immune checkpoint inhibitors also show promising results in previously treated advanced NSCLC, with pembrolizumab greatly improving overall survival compared with docetaxel in the KEYNOTE-010 study. (36)

Stage IV patients with a poor performance status or contraindications for immunotherapy can be offered a palliative chemotherapy. (10)

An important aspect of therapy is the early integration of palliative care for patients in an incurable metastatic stage of the disease. Palliative care helps with managing the burden of the physical symptoms but also addresses psychological, psychosocial, and spiritual concerns. Palliative care can also assist in coordinating care and helping with decision-making on treatment options. (10) For NSCLC, studies have shown that early palliative care improves patient's quality of life and mood. (37)

1.1.7 Prognosis

The prognosis of lung cancer remains poor. In 2019 , the five-year survival rate across all stages and histological subtypes was 22% in Austria. (1) The five-year-survival rate depends on the stage at diagnosis, the patient's age, and the tumour's histopathological type. (10)

According to the seventh edition of the TNM classification for lung cancer, the five-year survival rate for stage IA patients is 82%, for stage IB patients it is 66%, for stage IIA patients it is 52%, for stage IIB patients it is 47%, for stage IIIA patients it is 36%, for stage IIIB patients it is 19%, and for stage IV patients it is 6%. (31)

1.2 Mechanisms of PD-1 and PD-L1 inhibitors

During the development of cancer, cells undergo a range of genetic and epigenetic changes. These changes result in neoantigens, which the immune system can detect. It is constantly screening the body for neoplastic cells and destroying them. This is mostly accomplished by effector CD8+ T cells, which bind to the major histocompatibility complex (MHC I) on tumour cells and then release cytotoxic granules which kill the tumour cell. T cell activation and response is highly regulated by inhibitory and activating pathways, which are also called immune checkpoints. Normally, these pathways enable the protection of healthy cells and promote self-tolerance. Tumours can express inhibiting immune checkpoint proteins and thus escape being eliminated by immune cells. (38-40)

The PD-1 receptor and its ligands, PD-L1 and PD-L2, are transmembrane proteins that form the starting point to the inhibitory PD-1 pathway. In the PD-1 pathway cytotoxic CD8+ T cells in the tumour microenvironment are the main target, although PD-1 is broadly expressed on other activated immune cells, like B cells and macrophages. Priming of T cells in lymphoid tissues requires the presentation of tumour antigens by antigen presenting cells (APC) on a major histocompatibility complex (MHC), which binds to the T cell's T cell receptor (TCR). CD4+ T cells provide cytokines to help the priming and the clonal expansion of CD8+ cytotoxic T cells. CD8+ T cells are activated through co-stimulatory pathways and then proliferate, mature and travel to the site of the tumour. Within a short amount of time, those T cells also express PD1 and secrete cytokines, most importantly IFNγ, which activates tumour killing by macrophages and induces PD-L1 expression in those same macrophages as well as in tumour cells. This is called adaptive immune resistance of the tumour. This concept was first used by Drew Pardoll, and it explains how immunogenic tumours evade immune response without systemic immunosuppression. (39, 41, 42) In some tumours, PD-L1 overexpression is a result of constitutive oncogenic signalling in the tumour cell. Genetic alterations as well as epigenetic deregulation of PD-L1 also play a part. Some cancers also lose non-silent point mutations and therefore become less immunogenic. This is called innate immune resistance of the tumour. (39, 43, 44) The detailed mechanisms of PD-L1 expression on tumour and immune cells are not fully understood yet but are known to be complexly influenced by the tumour microenvironment. Apart from the above-mentioned mechanisms, other cytokines have been shown to upregulate PD-L1 expression, such as IL-1α, IL-10, IL-27, and IL-32γ.

(44, 45)

When effector CD 8+ T cells bind to PD-L1, this impairs their ability to proliferate, survive and produce cytokines. (45) When regulatory T cells bind to the ligand, this promotes both their induction and function, and they then further inhibit immune response. (46)

In short, tumours overexpress PD-L1 to evade attack of T cells and promote tolerance. Blocking this pathway with monoclonal antibodies binding to either the ligand or receptor can shift the tolerance between a tumour and a patient's immune system to the immune system actively working against the tumour again. This has been shown in a variety of cancers, including melanoma, lung cancer and Hodgkin's lymphoma. (47-49) This was thought to be primarily achieved through an activation of effector T cells and an inhibition of regulatory T cells, but as mentioned above, PD1 is widely expressed on range of cells and other mechanisms might help elicit an antitumour immune response. Macrophages in the tumour environment have been shown to express PD-1, and mouse models suggest that the PD-1 – PD-L1 pathway inhibits macrophage phagocytosis. (50) Natural killer (NK) cells also play an important role in destroying tumour cells. When NK cells bind to PD-L1 their cytolytic activity is inhibited. (51) When the PD-1/PD-L1 pathway is blocked, macrophages and NK cells are activated again and can support an anti-tumour response.

B cells in the tumour microenvironment may play an important role in tumour immunity, although there are conflicting findings on whether they are tumour-promoting or have antitumour properties. Both may be true, keeping in mind that B cells are heterogenous population with varying functions, and the balance between these subsets may be what is important to the overall effect on the modulation of cytotoxic T cell activity. (52) There is some evidence that a higher amount of B cells in association with tertiary lymphoid structures in the tumour microenvironment are predictive of the response to immune checkpoint therapy. Memory B cells may be acting as antigen presenting cells, and other B cells may secrete cytokines, provide co-stimulation, produce antibodies against the tumour, and work together with other immune cells to drive T cell activation. (52-54)

1.3 Side effects of immune checkpoint inhibitors

Immune checkpoint inhibitors can cause severe adverse events when used for the treatment of NSCLC. Adverse events in cancer clinical trials are graded according to the Common Terminology Criteria for Adverse Events (CTCAE), with grades referring to the severity of the adverse event. (A table explaining the grading of adverse events in clinical trials can be found in the appendix.) (55)

For example, in the KEYNOTE-010 study 13%-16% of patients treated with pembrolizumab developed a grade 3-5 adverse event depending on dosage. (47) In the CheckMate 017 study, 7% of patients who received nivolumab developed a grade 3-4 adverse event. (56) In the OAK trial, 15% of patients treated with atezolizumab developed grade 3-4 adverse events. (57) In the PACIFIC trial, 29.9% of patients treated with durvalumab developed a grade 3-4 adverse event. (58) In a pooled analysis, the incidence of all adverse events was 66.4% for atezolizumab, 71.8% for nivolumab, and 75.1% for pembrolizumab. (59) This shows adverse events are not a rare occurrence and affect a significant number of patients treated with immune checkpoint inhibitors.

Most of the adverse events are mild and present within the first few weeks of treatment. Some of the most common adverse events are pneumonitis, diarrhoea, nausea, colitis, decreased appetite, fatigue, rash, asthenia, musculoskeletal pain, pneumonia, and anaemia, but any organ can be affected, and this also differs between the individual immune checkpoint inhibitors. It is still not clear or predictable which patients will have an adverse event and which not. (47, 56-58, 60)

The pathophysiological mechanisms leading to adverse events are not fully understood yet. Most are considered immune-related adverse events, that happen because of on an overactivation of the immune system. It is thought that autoreactive T cells get activated and then attack normal tissues. In addition, there may be cross-reactivity between T cells attacking an antigen in the tumour and T cells attacking a similar antigen in normal tissue. Furthermore, PD-1 and PD-L1 are involved in regulating humoral immunity and self-tolerance, and a blockade might lead to an increase in pre-existing autoantibodies. (61) An increased level of inflammatory cytokines may be involved as well, with evidence that the blockade of interleukin-6 can be used to treat immune-related adverse events. (62) There is also evidence of an increase in mucosal IL-1β in patients who developed colitis. (63) Apart from the immune-related mechanisms described above, it has also been suggested that the

monoclonal antibodies themselves play a role in the development of adverse events by causing compliment-mediated injury. (64) Gut microbiota has also been suggested as an influential factor on the toxicity of immune checkpoint inhibitors. (63)

Fatal adverse events have been observed, although the risk remains generally lower than with conventional therapies. Anti-PD-1 therapies had a 0.36% fatality rate, and anti-PD-L1 therapies had a 0.38% fatality rate. These fatalities were most related to pneumonitis, hepatitis, colitis, and neurologic events (e.g., encephalitis). Fatalities occurred early after therapy initiation. (65)

Immune-related adverse events are usually managed by delaying the next administration of the ICI or with the use of cortisone or other immunosuppressants. For more severe cases a multidisciplinary management can be beneficial. It is still up for debate whether temporary immunosuppression required for the treatment of adverse events has a negative impact on the outcome of therapy. (61)

1.4 Predictive biomarkers for immune checkpoint inhibitor therapy in lung cancer

Considering the potentially severe side effects, the risk of ineffective treatment with response rates of around 20% in unselected patient cohorts (56, 57, 66), and the high cost of immune checkpoint inhibitor therapy, successfully predicting outcome is of utmost importance to ensure patients benefit from treatment. Several biomarkers have been suggested and established for predicting the response to immune checkpoint therapy, but they all come with their own flaws and difficulties. There is also a range of investigational biomarkers. (40, 67)

1.4.1 PD-L1 expression

PD-L1 expression within tumours has long served as a criterion for selecting patients suitable for PD-1 and PD-L1 therapy. PD-L1 is assessed using immunohistochemistry (IHC) on a tissue sample. It is typically quantified as the percentage of tumour cells expressing PD-L1, known as the tumour proportion score (TPS). (6, 68) An expression of at least 50% is considered predictive of positive response to immunotherapy, although the exact cut-off varies between 1-50%. (26, 33)

Whilst PD-L1 expression is associated with a positive outcome in most studies and is therefore the one most commonly used to support treatment decisions (10), some patients with expression below the threshold of 1% or without any PD-L1 expression still seem to profit from ICI therapy. For example, patients with metastatic NSCLC still profited from pembrolizumab in combination with chemotherapy irrespective of PD-L1 status. (56, 66, 69)

A significant challenge with this marker is the considerable intratumour variability in PD-L1 expression, leading to potential non-representativeness of biopsies for the entire tumour. (70) Especially lung adenocarcinomas often present a great spatial heterogeneity of PD-L1 expression. (67) However, there is some evidence suggesting a high concordance between cytological smears from fine needle aspiration, tissue cores and whole sections. (71)

PD-L1 expression can also vary between the primary tumour and metastases, with the metastatic sites often expressing less or no PD-L1. (72) The site of metastasis seems to matter, with bone and brain metastases showing the lowest PD-L1 expression and adrenal,

liver and lymph node metastases showing the highest expression, which might also impact treatment outcome. (73)

PD-L1 expression status appears to often change after treatment with immunotherapy or chemotherapy, with PD-L1 expression often dropping after previous ICI therapy and increasing after cytotoxic treatment. (73, 74) However, since PD-L1 is a tissue-based marker, a longitudinal assessment would require re-biopsy, which is invasive and costly. (68)

Moreover, there is notable variability depending on the assay utilized to assess PD-L1 expression. For the many different assays available there is a lack of prospective clinical trials showing clinical validity. (67) Furthermore, IHC-based markers have the disadvantage of being subjective to the person assessing the sample. (68)

1.4.2 Tumour mutational burden (TMB)

Tumour mutational burden is a way to quantitatively measure the total number of somatic nonsynonymous mutations per coding area of a tumour genome. Tumour mutational burden generally requires tissue of the tumour and can be analysed from whole-exome sequencing as well as gene-targeted next generation sequencing. (75) However, there is no standardized panel or method when it comes to the next generation sequencing. (67, 68) Tumour mutational burden is typically higher in smokers, who display a complex mutational signature and often have tumour suppressor gene alterations. This results in a higher likelihood of the tumour cells expressing neoantigens that can be detected by the immune system, which in turn triggers a tumour cell killing, cytotoxic response. This is a likely explanation for a greater success of ICI therapies in tumours with high TMB. However, TMB can only be a surrogate marker for this complex immunological process, as it does not predict the antigenicity of the neoantigens, the ability of the tumour to express antigens or the immune system's ability to recognise the antigens and to adequately respond. (67, 68, 76, 77)

In the US, the FDA has approved pembrolizumab use for any microsatellite-stable, solid, TMB-high tumours that have progressed, with the definition of TMB-high being at least 10 mutations per megabyte based on a study with 9 different tumour types. However, NSCLC

was not included in this study. Furthermore, TMB was a predictor of progression-free survival and not overall survival in this study. (68, 78, 79)
It is questionable whether such a cut-off can be used as a pan-cancer marker and whether this decision is relevant for lung cancer, as there is evidence that the TMB cut-offs associated with better outcome can vary between cancer types. (80, 81)

For NSCLC, TMB has been shown to be an inconsistent predictor around the median but a better predictor at the 80th to 90th percentile cut-offs. (80)
In the CheckMate trials evaluating nivolumab plus ipilimumab in NSCLC, TMB was associated with improved overall response irrespective of PD-L1 expression, and the cut-off for this was defined as >10 mutations per megabase. (82, 83) For pembrolizumab, high TMB was associated with improved outcomes in the KEYNOTE trials; however, if pembrolizumab was combined with chemotherapy, TMB was not predictive of outcome, possibly limiting TMB's utility in this setting. (76)

PD-L1 and TMB lack significant association in several studies, suggesting they might be independent and complimentary biomarkers. TMB might be used to identify patients with high PD-L1 which would benefit from immunochemotherapy instead of immunotherapy alone. (67, 76, 77) There is some evidence to suggest that the combination of PD-L1 and TMB might be useful for identifying long-term responders. (84)

There are several trials evaluating blood-based TMB, which is measured using next generation sequencing-based panel tests for detecting mutations in cell-free DNA. There is some data suggesting blood based TMB is associated with better clinical outcomes, however, blood TMB still seems to be a weak predictor on its own, and further research will be necessary regarding the assays used. It could hold some benefit if there are not sufficient tissue samples for evaluation of tissue-based biomarkers like PD-L1. (76, 77)

1.4.3 Microsatellite instability

Microsatellite instability results from deficient mismatch repair, which drives mutations in microsatellite regions and results in a high mutational load, which generates neoantigens. It can be viewed as a subgroup of TMB and has similar challenges regarding the actual antigenicity od the neoantigens and the patient's immune system being able to detect and respond to the neoantigens. Additionally, only a small subset of patients have MSI-high

tumours, which makes this biomarker not broadly applicable. MSI can be determined using PCR or IHC, however, there is no standardised testing method. It is not commonly used in lung cancer. (68)

1.4.4 Tumour genomic driver mutations

With multiple targeted treatment options available for NSCLC, genomic driver mutations play an important role as predictive biomarkers in the clinical setting and are routinely analysed in patients. They may also play a role as predictive markers for ICI therapy, even though the use of immunotherapy in patients with oncogenic driver mutations remains controversial. (85) Genomic mutations can be detected via NGS panels or exome sequencing. The detection of specific mutations via IHC is also possible. All these methods require a sufficient amount of tissue samples, which is lacking in about a third of lung cancer patients. Blood-based circulating tumour DNA NGS can also be an option and results are promising, but further research will be necessary to establish this either as a stand-alone method or in combination with tissue-based methods. (67, 86)

In non-smokers EGFR mutations and ALK fusions are common. These mutations suggest a lower mutational burden and therefore a lower immunogenicity of the tumour, resulting in a lower response to ICIs. Overactivation of EGFR also induces CD73 expression, which is associated with a low PD-L1 expression and lower INF gamma signature, which results in lower T cell activation, resulting in lower efficacy of ICI therapy. Similarly, ROS1 fusions are also associated with poor response to ICIs, but due to the rarity of this mutation data is lacking. (67, 87, 88)

KRAS gain-of-function mutations are the most common oncogenic driver mutations in lung adenocarcinomas and are found in both smokers and never-smokers. They are generally associated with a greater PD-L1 expression and a positive response to ICI therapy. (67, 85, 87, 88) A common co-mutation is loss of TP53 function, which is associated with a high expression of PD-L1 and high TMB, and therefore a positive response to ICIs. However, two other common co-mutations, STK11 and KEAP1, are associated with a lower likelihood of response. Loss of STK11 function has been associated with a high TMB, however, it has been also associated with lower immune surveillance of the tumour and deficient MHC I antigen-presentation. STK11 is not yet

routinely used as a negative predictive marker for ICI therapy due to the lack of prospective studies. (67, 87-89)

BRAF mutations, which are more frequent in smokers, and BRAF V600E mutations, which are more frequent in never-smokers and females, have been associated with better outcomes after ICI therapy, probably due to a high expression of PD-L1. However, the amount of data on this is small. (85, 88, 90) Similarly, MET exon 14 skipping mutations show a variable response to ICI, but are also associated with a high PD-L1 expression. (67)
HER2/ERBB2 mutated tumours have been shown to respond better to ICI therapy than those with EGFR mutations. However, they do not show a better response than unselected populations. (67, 88)
The data on RET fusions and the efficacy of ICI therapy is somewhat conflicting, but overall points to a low response to ICI therapies. (85, 88)
In one large multicentre study, no significant differences in genomic mutations were found between short-term and long-term responders with NSCLC. (84)

Overall, genomic profiling for ICI therapy falls short of other available biomarkers of response. At this point, they could potentially be useful for selecting patients suitable for salvage ICI therapy after targeted treatment options have been exhausted. (67)

1.4.5 Tumour immune microenvironment

The tumour microenvironment a current area of interest in the search for a new biomarker. Factors like tumour and immune gene mutation signatures, tumour loss of antigen presentation and immune cells infiltrating the tumour have been investigated as predictive biomarkers. NGS of patient tissue samples can be used to assess these factors as well as spatial imaging analyses. (68)

Considering the mechanisms of ICI therapies, infiltrating CD8+ T cells seem a good candidate for a predictive biomarker, as they indicate a state of IFNγ driven immune resistance in the tumour. However, it has proven challenging to establish a predictive marker based on CD8+ cell number, as these cells can be in different physiological states. (67)
It has been suggested that the absence of PD-1 co-expression indicates naïve or resting T cells that can still be activated and predicts improved outcome after ICI therapy. (91) It has

been suggested that a T cell exhaustion phenotype is beneficiary, hypothesizing that ICI therapy could reverse this state and trigger a new cytotoxic response (67, 92), however, another hypothesis is that the T cell exhaustion phenotype could be a factor for resistance against ICI, because there are too many apoptotic and dysfunctional CD8+ T cells crowding other T cell populations out. Further research will be necessary before T cells can be a clinically useful biomarker. (67, 93)

IFNγ induces the expression of MHC I and II on tumour cells. Tumours usually have a range of mechanism to down regulating the expression MHC I, for example mutations or loss of the HLA genes encoding the MHC or its co-factors, thus being able to avoid detection by CD8+ cytotoxic T cells. Even though mutations in HLA genes are not common in lung cancer, loss of heterozygosity is reported in about 40% of patients with NSCLC. The loss of MHC I expression can be seen in about a third to a half of tumour samples in NSCLC, which is associated with a lower infiltration of CD8+ T cells and a worse overall prognosis. Even though large pan cancer studies have associated HLA-high gene expression to a positive response to immunotherapy, MHC expression has not been sufficiently investigated as a predictive biomarker . (67)

1.4.6 CRP

Inflammation has been long known to play an important role in the development and progression of cancer. In a state of chronic inflammation, immune cells can release a range of reactive oxygen and nitrogen species that can cause DNA damage. Cytokines and proteins present in an inflamed microenvironment contribute to cancer progression by promoting cell proliferation, angiogenesis, and cancer cell migration. (38, 94-96)
Considering the mechanisms of ICIs, it is possible that peripheral blood-based markers of inflammation could predict outcome and response to these treatments.

C-reactive protein (CRP) is an acute-phase protein mostly synthesised by the liver and regulated mainly by interleukin (IL)-6 and IL-1β. It is a general marker of inflammation in the body. Its baseline production in a healthy individual is influenced by both genetics and environmental factors (e.g. smoking). (38, 96)
CRP can exist in different isoforms. The pentameric isoform, consisting of five subunits, which is the form quantified in diagnostic tests, is less bioactive, and when binding to an

activated cell membrane, dissociates into a monomeric, modified isoform, which can insert into activated cell membranes and stimulates platelet and leukocyte responses. (97, 98) CRP is commonly elevated in cancer patients as an expression of systemic inflammation due to an immune attack on the neoplastic cells, in response to tumour cells secreting cytokines and chemokines that stimulate its production, and as an expression of tissue damage. Elevated CRP levels in cancer patients have been associated with poor prognosis in many studies. Furthermore, CRP levels correlate with tumour size and staging in lung cancer, signifying tissue-damage. (38, 95-98)

CRP has also been evaluated as a predictive biomarker for ICI therapy in several studies. (94, 99) One meta-analysis of 33 studies including a total of 6,124 patients showed that elevated CRP at baseline correlated with worse OS and PFS in 9 types of cancer treated with 6 different ICIs. The most common cut-off in these studies was 1 mg/dl (=10 mg/L). (100) In NSCLC, a bi-centre study by Riedl et al. showed that baseline CRP was a prognostic marker of overall survival (OS), progression-free survival (PFS). They also investigated CRP as a dynamic marker used to measure disease activity and treatment response. The study showed that a continued elevation of CRP during anti PD-(L)1 treatment indicates worse outcome and that an early decline in the first 8 weeks after the start of treatment predicts longer PFS. (94) Oya et al. found that elevated pre-treatment CRP (>10mg/L) was associated with worse PFS in patients with NSCLC treated with nivolumab. (101)

There are several hypothesizes as to why CRP might predict the outcome of ICI therapy, but the exact mechanisms are still unclear. In melanoma patients, CRP has been shown to inhibit the proliferation of peripheral blood CD4+ and CD8+ T cells. It upregulated their checkpoint protein expression, altered their phenotype, reduced the expression of IL-2 and IFNγ and inhibited TCR signalling. It also upregulated the expression of IL-1β in CD8+ T cells, which then in turn stimulates further CRP production by the liver. CRP also inhibited the expansion of Melan-A-antigen specific CD8+ T cells. Furthermore, CRP was shown to inhibit the activation and expression of co-stimulatory molecules on mature dendritic cells. In short, CRP seems to suppress adaptive immunity in peripheral blood T cells and dendritic cells. These effects directly affect the mechanism of ICIs. It remains to be seen whether these mechanisms are fully translatable to other types of cancer. (102)

In the TME, it seems like whilst CRP may help to retain leukocytes which aid tumour cell lysis early on in tumourigenesis, it might prolong immune recruitment and lead to a pro-inflammatory and pro-tumourigenic environment, however, further research will be necessary to fully understand its impact on the TME. (97)
Furthermore, CRP, which binds to endothelial and epithelial cells and proteins in the extracellular matrix (eg. fibronectin, collagen) may also play a role in activating the fibrinolytic-like response that are part of cancer growth. Monomeric CRP may stimulate a range of pro-inflammatory intracellular pathways like NFκB, which also facilitates cancer growth. (97)
As stated above, especially pentameric CRP is a marker of tissue-damage, and higher pre-treatment levels might indicate progressed disease, which might correlate with worse survival. (100)

The measurement of CRP is highly sensitive, specific, reproduceable, cost-effective, and is readily available in many clinical settings worldwide. (95)
Another benefit of CRP is that it is a blood-based biomarker that is less invasive and associated with less complications and pain than tissue-based markers. Furthermore, it is likely to be influenced by all tumour lesions and therefore reflects an average of all the tumour sites, whereas tissue -based markers only assess a specific site. This also makes an ideal dynamic marker to be measured repeatedly over the course of treatment. (68)
Overall, CRP appears to be a promising marker, but further research will be necessary to learn more about the mechanisms and to establish standardised cut-offs.

1.4.7 White blood cells

The neutrophile-to-lymphocyte ratio (NLR) is another blood-based marker of non-specific acute inflammation and immune activation and has been proposed as a predictive biomarker for ICI therapies. It is calculated by dividing absolute peripheral neutrophile count by absolute peripheral lymphocyte count from a routine lab. (103, 104) In solid tumours, it has been known as a prognostic marker of OS irrespective of treatment. (105)

Neutrophiles have long been linked tumourigenesis, as they have been shown to support angiogenesis and to activate proteolysis, which in turn stimulates the secretion of tumour promoting growth factors. They also secrete metalloproteinases which promote tumour cell dissemination by altering the extracellular matrix. They also produce reactive oxygen

species (ROS), which can cause DNA damage. Moreover, they recruit other immune cells to the site of the tumour. Immature neutrophiles known as G-MDSC (granulocytic myeloid derived suppressor cells) have immunosuppressive effects in the TME.
In contrast to that, neutrophiles have also been shown to kill tumour cells and to stimulate the T-cell mediated immune attack on tumour cells. It has been proposed that whether neutrophiles are anti-tumour or tumour promoting depends on the cytokines present in the TME. Another hypothesis is that there are subgroups of tumour promoting N2 neutrophils and anti-tumour N1 neutrophils. (104, 106, 107)

Peripheral neutrophile counts measured via NLR has been shown to correlate with the intratumour neutrophile population, so in a high NLR the neutrophiles are possibly hindering the immune attack on the tumour. Furthermore, a high NLR because of lymphopenia might also signal a worse response to anti-PD-1/PD-L1 therapy, as lymphopenia might suggest a worse cell mediated immunity and lymphocytes are an essential target structure of this treatment. (103) NLR values also correlate with values of immunosuppressive polymorphonuclear MDSCs, which comprise neutrophils and are associated with an advanced stage of disease. (104)

In NSCLC treated with PD1/PDL1 blockers, one meta-analysis of 15 studies including a total of 1700 patients concluded that a high NLR both pre- and post-treatment was associated with shorter PFS and OS. The most common cut-off was 5.0 and the median cut-off was 5.0. (103, 108) A study by Li et al. also showed NLR at baseline to be a predictor of OS, but also showed that change in NLR during treatment with ICIs to be a non-linear predictor of OS, with patients with a moderate decrease having the longest survival, and patients with a large increase or decrease having shorter OS. (109)

The exact mechanisms of why NLR might predict ICI response are still unclear. Given the complex interactions of neutrophils and the tumour, NLR has been criticised as an oversimplifying marker. The derived NLR includes the total peripheral white cell count into the equation and is calculated as absolute neutrophils divided by the difference of white blood cell count minus absolute neutrophils. It has been shown to be a predictive biomarker of ORR, PFS and OS in advanced NSCLC patients treated with pembrolizumab. (104, 110) A recent meta-analysis of 8 studies showed that elevated dNLR was associated with worse OS. (111)

Apart from NLR, absolute neutrophil counts have been investigated a biomarker as well. Elevated pre-treatment ANC has been associated with worse OS in several studies, with the most common cut-off being 6.0 10^9/L. (111) A study by Murakami et al. showed that a decrease in ANC after the has been associated with long-term survival in NSCLC patients treated with nivolumab. (112) Another study showed that neutrophils 8-12 weeks after initiation of treatment were associated with OS. (113) The exact mechanisms are unclear. It is possible that absolute neutrophil count could be associated with neutrophil count in the TME, similarly to NLR, however, this has not been confirmed yet. Furthermore, peripheral neutrophil count has not been validated.

Total leukocytes have been investigated as a predictive marker in a few studies, but the amount of data is small and somewhat inconclusive. However, the available studies show a tendency towards an increase of WBC at baseline being associated with worse OS. (111)

Eosinophils are another group of circulating immune cells that has been investigated as markers of response to ICIs in lung cancer, however, the amount of data available is small and controversial. One study suggested that an increase of eosinophils after initiation of treatment was predictive of response, whilst other studies did not show a significant association. (111, 113)

CRP, NLR and dNLR are widely investigated and promising markers of response to ICI therapy in NSCLC, they are not established in a clinical setting, despite being cost-effective and non-invasive. However, these markers have not been investigated for patients treated ICI in combination with chemotherapy, even though this has been a standard of care. Also they lack standardised cut-offs and have not been investigated in prospective studies. (111)

1.5 Aim of the diploma thesis

As described above, there is a great need for new biomarkers to personalise the use of immune-checkpoint inhibitors and target it towards patients who benefit from this treatment. The aim of the study was to evaluate the predictive value of a set of routine blood parameters measured at baseline and during treatment and to generate new hypotheses.

2 Methodology

A total of 36 patients with lung cancer (NSCLC) who were due to receive immunotherapy were recruited between April 2019 and October 2020 at the Division of Oncology or the Division of Pulmonology at the University Hospital Graz. One patient never received the immunotherapy and had to be excluded. 35 patients were included in the final analysis. Written consent was obtained from all participants. The study was approved by the local ethic committee.

Demographic characteristics as well as clinical and pathological data were obtained from electronic medical records. Laboratory data was obtained as part of routine clinical evaluation prior to the initiation of treatment and consecutively every three months as part of regular check-ups. All samples were analysed by the Graz university hospital's laboratory using the standard clinical testing methodology. CRP levels were assessed using a latex particle-enhanced immunological turbidimetric assay by Roche Diagnostics, with a range from 0.3 to 350 mg/L. Laboratory data was then collected from the university hospital's electronic medical records. All laboratory data was copied and pasted. The laboratory data was then checked for accuracy. This was done by checking the values for plausibility and then checking the lowest and highest value of each parameter as well as ten values picked at random.

The following parameters were included: leukocytes (10^9/L), erythrocytes (10^12/L), haemoglobin (g/dL), haematocrit (%), MCV (fL), MCH (pg), MCHC (g/dL), thrombocytes (10^9/L), MPV (fL), neutrophiles (%; 10^9/L), eosinophiles (%; 10^9/L), basophiles (%; 10^9/L), monocytes (%; 10^9/L), lymphocytes (%; 10^9/L), and CRP (mg/L). Neutrophile-lymphocyte ratio (NLR) was calculated by dividing absolute neutrophiles (10^9/L) by absolute lymphocytes (10^9/L). Due to the low number of patients left, only pre-treatment parameters and parameters three month after the initiation of treatment were included in the analysis.

Overall survival was the primary endpoint and was calculated in months from the date of initial treatment to date of death from any cause. The date of the last follow-up was 22 February 2022. Survival was extracted from the patient record.

Statistical analysis was performed with IBM SPSS Statistics version 29 (SPSS Inc., Chicago, IL, USA). Median follow-up time was calculated with a reverse Kaplan-Meier analysis according to Schemper and Smith. (114) A p-value < 0.05 was considered significant. Analysis was performed for the whole patient cohort at first. A subgroup analysis was performed for all patients receiving pembrolizumab, because of differences in eligibility criteria between the individual drugs (for example PD-L1 expression levels). For the other treatment groups, results were not calculated because of small group size.

As a first step, univariate Cox-regression analysis was performed for each laboratory parameter to determine the influence on overall survival (OS). Hazard ratios (HR) estimated from the Cox proportion analysis were reported with corresponding 95% confidence intervals (CI). Parameters with a significance of 0.05 or below were further analysed in a multivariable Cox regression.

Since cut-offs are not known for the purpose of predicting response to immune checkpoint inhibitors, for each parameter patients were split into two groups using the median. Overall survival rates (OS) were calculated for each parameter using Kaplan-Meier curves, and a log-rank test was applied for statistical comparison between curves.
Furthermore, a scatterplot was used to visualise overall survival and CRP.

3 Results

3.1 Characteristics of cohort

Thirty-six lung cancer patients with intended immune checkpoint inhibitor therapy were recruited. One patient never received ICI therapy and had to be excluded, and a total of 35 patients were included in the present analysis. The median age of the patients was 63 years. 57.9% of patients were male and 42.1% were female. All patients were diagnosed with NSCLC. Twenty-four patients had an adenocarcinoma, ten a squamous cell carcinoma, and one a large-cell carcinoma. Twenty-four patients were treated with pembrolizumab; five were treated with nivolumab; three were treated with atezolizumab; and three were treated with durvalumab.

Criterion	**Value**
Number of Patients	35
Median Age (Range)	63 (46-81)
Sex	
Male	20 (57.9%)
Female	15 (42.1%)
Histological Type	
Adenocarcinoma	24 (68.6%)
Squamous Cell Carcinoma	10 (28.6%)
Large Cell Carcinoma	1 (2.8%)
PD-(L)1 Inhibitor	
Pembrolizumab	24 (68.6%)
Nivolumab	5 (14.3%)
Atezolizumab	3 (8.6%)
Durvalumab	3 (8.6%)

Table 1: Characteristics of the entire cohort

3.2 Results for entire cohort

The median follow-up time was 24 months. During the entire follow-up period, a total of 23 patients died (65.7%). Estimated OS after 12 months was 45.7%, and OS after 24 months was 31.2%, according to a Kaplan-Meier analysis.

In a univariate Cox-regression analysis, increased pre-treatment CRP ($p = 0.047$; HR 1.010, 95% CI 1.000 – 1.021) was significantly associated with shorter OS, as well as WBC three months after the initiation of treatment (HR 1.177; CI 95% 0.373 – 1.948; $p = 0.025$) and ANC three months after the initiation of treatment (HR 1.040; 95% CI 1.051 – 1.498; $p = 0.012$). None of the other remaining laboratory pre-treatment parameters analysed were significantly associated with OS. Patient age at the start of therapy and patient sex were both not associated with OS.

The median pre-treatment CRP was 14.00 mg/dL. Kaplan-Meier analysis for pre-treatment CRP showed a median OS of 19.0 months (95% CI 10.4 – 27.6) for CRP ≤14.00 mg/L and a median OS of 5.0 months (95% CI 2.4 – 7.6) for CRP >14.00 mg/L. The p-value was 0.15. The total number of events in the CRP ≤14.00 mg/L group was 9/17, the total number of events in the CRP >14.00 mg/L group was 12/16. In the pre-treatment CRP ≤14.00 mg/L group, the OS after 12 months was 64.7%, and the OS after 24 months was 44.1%. In the pre-treatment CRP >14.00 mg/L group the OS after 12 months was 31.3% and the OS after 24 months was 20.8%.

An exploratory ROC analysis of pre-treatment CRP showed that the median of 14.00 mg/dl was the ideal cut-off (area under the curve: 0.69).

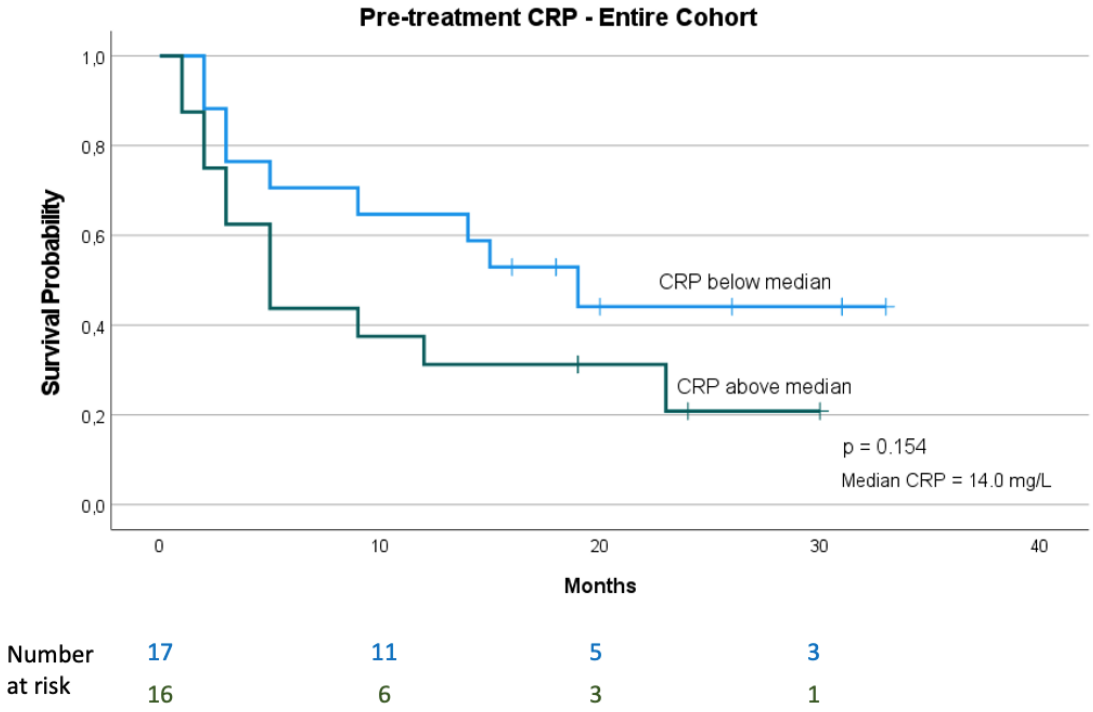

Figure 1: Kaplan-Meier curve – pre-treatment CRP for entire cohort

In a univariate Cox-regression analysis of parameters three months after the initiation of treatment, white blood cell count (WBC, leukocytes) (HR 1,177, 95% CI 1.020 – 1.357, p=0.025) and absolute neutrophil count (ANC) (HR 1.254, 1.051 – 1.498, p=0.012) were significantly associated with OS. Neither CRP after three months nor $\Delta CRP_{1,2}$ was significantly associated with OS.

The median WBC (leukocytes) after three months was 8.565 10^9/L. Kaplan-Meier analysis for WBC after three months showed a median OS of 19.0 months (95% CI 10.4 – 27.7) for WBC below the median and a median OS of 5.0 months (95% CI 0.0 – 12.3) for WBC above the median. The p-value was 0.365.
The median ANC (neutrophils) after three months was 6.65 10^9/L. Kaplan-Meier analysis for ANC after three months showed a median OS of 19.0 months (95% CI 10.4 – 27.7) for ANC below the median and a median OS of 5.0 months (95% CI 0.0 – 12.3) for ANC above the median. The p-value was 0.365.

In an additional Kaplan-Meier analysis, the estimated median OS differed depending on the immune checkpoint inhibitor used. The median OS for Pembrolizumab was 9.0 months (95% CI 0.0 – 18.6), the median OS for Nivolumab was 12 months (95% CI 0.00 – 31.3),

the median OS for Atezolizumab was 3.0 months (CI not applicable), and the median OS for Durvalumab was 19.0 months (CI not applicable). This was not statistically significant (p=0.162).

In a multivariable Cox regression including all parameters with p = < 0.05 and age as a probable influential factor, none of the parameters remained significant. However, the hazard ration for pre-treatment CRP (HR 1.010; 95% CI 0.950 – 1.026; p=0.208) remained the same as in univariate Cox regression.

Parameter at Baseline *(N = 35, if not otherwise specified)*	**HR**	**95% CI**	**p-Value**
Sex	0.852	0.373 – 1.948	0.705
PD-L1 expression n = 28	0.996	0.982 – 1.010	0.549
Age	0.990	0.950 – 1.032	0.627
WBC	1.051	0.926 - 1.192	0.440
RBC	1.059	0.599 – 1.871	0.844
Hemoglobine	0.973	0.818 – 1.157	0.753
Hematocrite	0.990	0.926 - 1.057	0.758
MCV	0.906	0.812 – 1.011	0.078
MCH	0.817	0.626 – 1.066	0.137
MCHC	1.023	0.724 – 1.455	0.899
Platelets	1.001	0.988 – 1.005	0.520
MPV	1.043	0.636 – 1.713	0.867
% Neutrophils	1.020	0.968 – 1.073	0.463
Absolute Neutrophil Count	1.040	0.898 – 1.205	0.598
% Eosinophiles	1.064	0.858 – 1.321	0.572
Absolute Eosinophil Count	3.950	0.435 – 35.898	0.222
% Basophiles	0.727	0.299 – 1.771	0.438
Absolute Basophil Count	13.747	0.003 – 63207.749	0.542
% Monocytes	0.944	0.812 – 1.098	0.453
Absolute Monocyte Count	0.907	0.275 – 2.992	0.873
% Lymphocytes	0.962	0.893 – 1.037	0.312
Absolute Lymphocyte Count	0.850	0.448 – 1.613	0.619
CRP n = 33	1.010	1.000 – 1.021	0.047
NLR	1.007	0.871 – 1.165	0.924

Table 2: Hazard ratios and 95% CIs calculated with univariate Cox regression of pre-treatment parameters for the entire cohort

Parameter at 3 months *(N = 28, if not otherwise specified)*	**HR**	**95% CI**	**p-Value**
WBC	1.177	1.020 - 1.357	0.025
RBC	0.815	0.391 – 1.699	0.815
Hemoglobine	0.793	0.594 – 1.057	0.114
Hematocrite	0.936	0.847 - 1.036	0.201
MCV	0.959	0.886 – 1.039	0.309
MCH	0.897	0.766 – 1.050	0.177
MCHC	0.828	0.612 – 1.120	0.221
Platelets	1.000	0.996 – 1.004	0.959
MPV	1.232	0.608 – 2.499	0.563
% Neutrophils	1.084	0.984 – 1.193	0.101
Absolute Neutrophil Count	1.254	1.051 – 1.498	0.012
% Eosinophiles	1.119	0.939 – 1.334	0.208
Absolute Eosinophil Count	2.197	0.909 – 5.331	0.080
% Basophiles	1.025	0.439 – 2.391	0.954
Absolute Basophil Count	41.476	0.007 – 259316.983	0.404
% Monocytes	0.798	0.620 – 1.026	0.079
Absolute Monocyte Count	1.872	0.298 – 11.749	0.504
% Lymphocytes	0.937	0.859 – 1.022	0.145
Absolute Lymphocyte Count	0.788	0.324 – 1.917	0.599
CRP n = 27	1.002	0.989 – 1.016	0.732
NLR	1.096	0.981 – 1.225	0.103
$\Delta CRP_{1,2}$	1.004	0.991 – 1.018	0.521

Table 3: Hazard ratios and 95% CIs calculated with univariate Cox regression of parameters three months after the initiation of treatment for the entire cohort

Parameter	HR	95% CI	p-Value
Age	0.989	0.932 – 1.050	0.721
CRP_1	1.010	0.994 – 1.026	0.208
WBC_2	0.649	0.252 – 1.668	0.369
ANC_2	2.190	0.669 - 7.168	0.195

Table 4: Hazard ratios and 95% CIs from multivariable Cox regression analysis of the entire cohort

3.3 Characteristics of pembrolizumab subgroup

Twenty-four patients were treated with pembrolizumab. The median age of the patients was 63.5 years. 62.5% of patients were male and 37.5% were female. Sixteen patients were treated for adenocarcinoma, 7 for squamous-cell carcinoma, and one patient for large-cell carcinoma.

Criterion	Value
Number of Patients	24
Median Age (Range)	63.5 (45-80)
Sex	
Male	20 (57.9%)
Female	15 (42.1%)
Histological Type	
Adenocarcinoma	16 (66.7%)
Squamous Cell Carcinoma	7 (29.2%)
Large Cell Carcinoma	1 (4.2%)

Table 5: Characteristics of the pembrolizumab subgroup

3.4 Results for pembrolizumab subgroup

The median follow-up time was 24 months. During the entire follow-up period, a total of 15 patients died (62.5%). The OS after 12 months was 45.8%; the OS after 24 months was 37.5%.

In a univariate Cox-regression analysis, elevated pre-treatment CRP was associated with shorter OS (HR 1.019; 95% CI 1.006 – 1.032; p=0.003), as well as CRP three months after the initiation of treatment (HR 1.030; 95% CI 1.004 – 1.057; p=0.025), WBC three month after the initiation of treatment (HR 1.205; 95% CI 1.007 – 1.443; p=0.042), ANC three month after the initiation of treatment (HR 1.287; 95% CI 1.028 – 1.61; p=0.028), % eosinophiles three months after the initiation of treatment (HR 1.219; 95% CI 1.012 – 1.469; p=0.037), and AEC three month after the initiation of treatment (HR 2.906; 95% CI

1.086 – 7.774; p=0.034). None of the other remaining laboratory pre-treatment parameters analysed were significantly associated with OS. Patient age at the start of treatment and patient sex were not influential factors on OS in univariate Cox-regression analysis.
In a multivariable Cox regression including parameters with statistical significance in univariate analysis as well as patient age, pre-treatment CRP (HR 1.030; 1.006 – 1.055; p=0.015) as well as CRP after three months (HR 1.094; 95% CI 1.023 – 1.170; p=0.009) remained significant predictors of overall survival.

For CRP before treatment, the median was 13.2 mg/L. Kaplan-Meier analysis of pre-treatment CRP showed a median OS of 15.0 months (95% CI not applicable) for CRP below the median and a median OS of 5.0 months (95% 0.0 – 12.5) for pre-treatment CRP above the median. The p-value was 0.151. The total number of events was 6/12 in the CRP ≤13.2 mg/L group, and 8/11 in the CRP >13.2 mg/dL group.

In the pre-treatment CRP ≤13.2 mg/L group, the OS after 12 months was 66.7% and the OS after 24 months was 50.0%. In the pre-treatment CRP >13.2 mg/dL group, the OS after 12 months was 27.3% and the OS after 24 months was 27.3%.

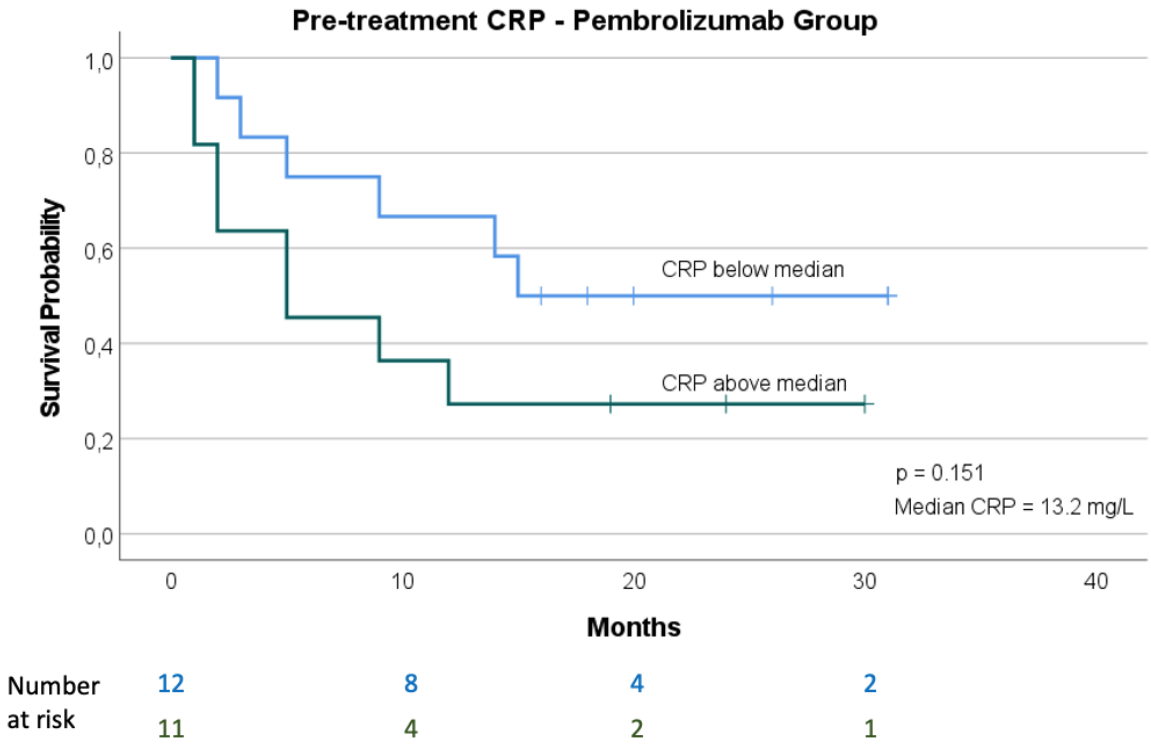

Figure 2: Kaplan-Meier curve– pre-treatment CRP for Pembrolizumab group

An exploratory ROC analysis was performed for pre-treatment CRP, and it showed that the ideal cut-off was 8.45 mg/L (AUC: 0.746). A Kaplan-Meier analysis with this cut-off did not reach the median OS for CRP below 8.45 mg/L and a median OS of 5.0 months (95% 2.7 – 7.3) for pre-treatment CRP above 8.45 mg/L. The p-value was 0.01. The total number of events was 3/9 in the CRP <8.45 mg/L group, and 11/14 in the CRP >8.5 mg/L group.

In the pre-treatment CRP ≤8.45 mg/L group, the OS after 12 months was 88.9 % and the OS after 24 months was 66.7 %. In the pre-treatment CRP >8.45 mg/dL group, the OS after 12 months was 21.4% and the OS after 24 months was 21.4%.

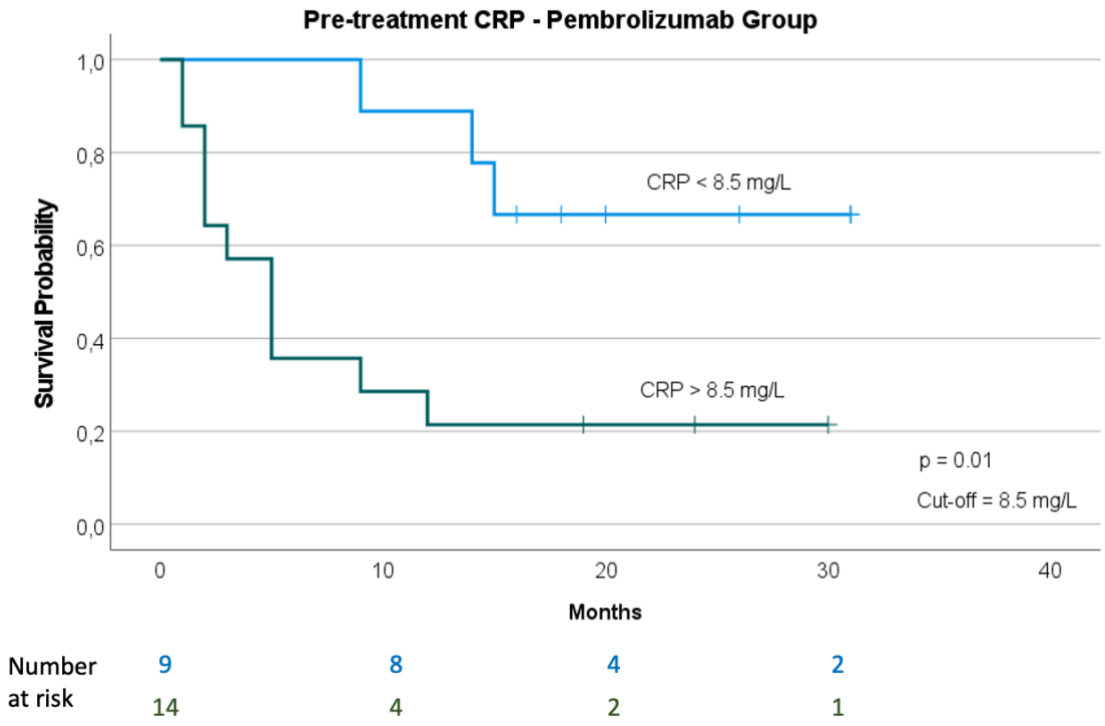

Figure 3: Kaplan-Meier curve – pre-treatment CRP with new cut-off for Pembrolizumab subgroup

To further visualize pre-treatment CRP within the Pembrolizumab subgroup, a scatterplot was generated, showing the deceased and censored patients in relation to survival time and pre-treatment CRP.

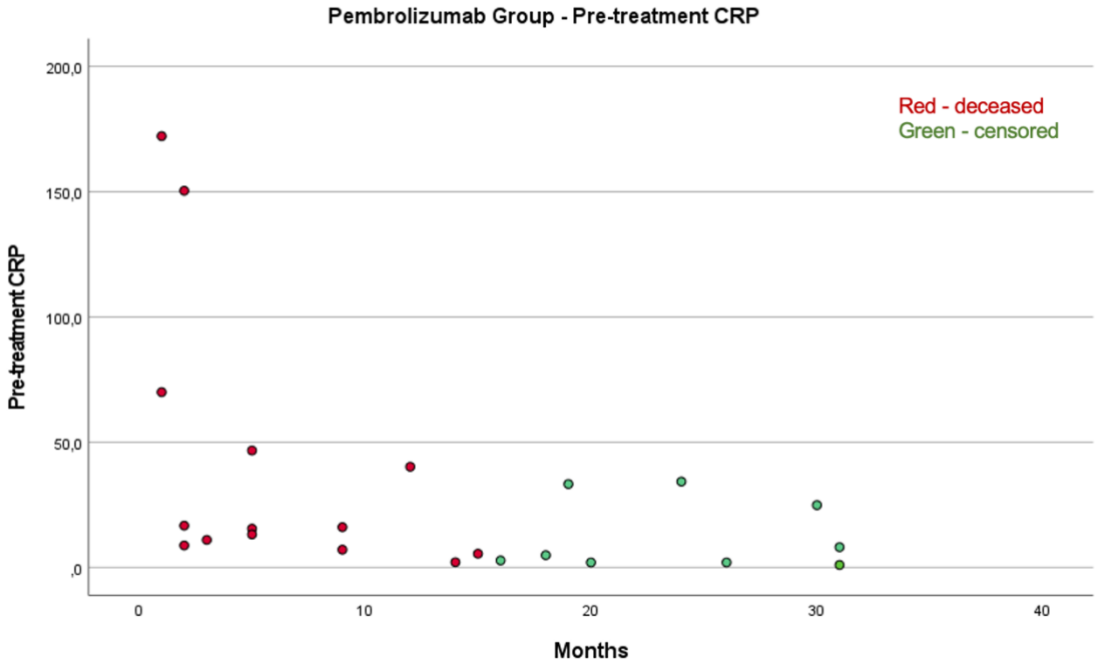

Figure 4: Scatterplot with pre-treatment CRP in the Pembrolizumab subgroup

For CRP three months after the initiation of treatment (CRP_2), the median was 6.35 mg/dL. In a Kaplan-Meier analysis for CRP three months after the initiation of treatment, the CRP ≤6.35 mg/dL group did not reach the median OS, whereas the median OS for the CRP >6.35 mg/dL was 6.0 months (95% CI 3.1 – 8.9). The p-value was 0.001. The total number of events in the CRP ≤6.35 mg/dL group was 2/9, and 9/9 in the CRP >6.35 mg/dL group. In the CRP_2 ≤6.35 mg/dL group, the OS after 12 months was 88.9% and the OS after 24 months was 77.8%. In the CRP_2 >6.35 mg/dL group, the OS after 12 months was 11.1% and the OS after 24 months was 0.0%.

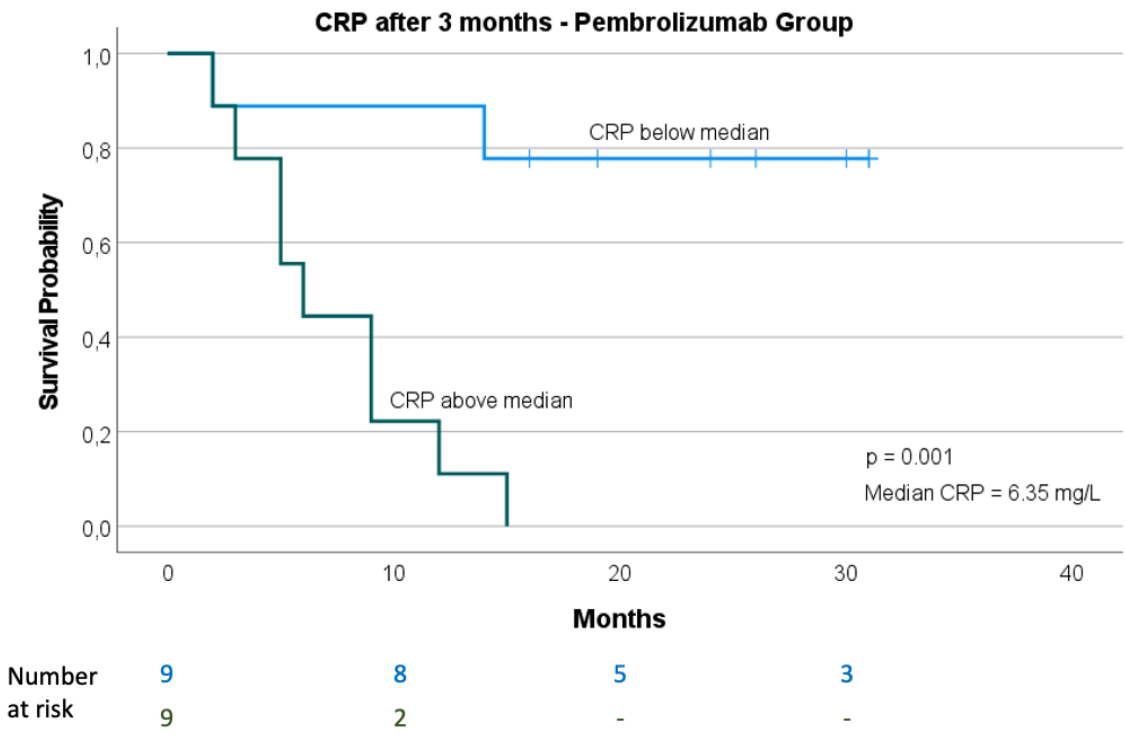

Figure 5: Kaplan-Meier curve – CRP three months after initial treatment for Pembrolizumab group

An exploratory ROC analysis for CRP_2 showed that the ideal cut-off was the median of 6.35 mg/L (AUC: 0.883).

For WBC three months after the initiation of treatment (WBC_2), the median was 9.65 10^9/L. Kaplan-Meier analysis of WBC_2 showed a median OS of 15.0 months (95% CI not applicable) for WBC_2 below the median and a median OS of 9.0 months (95% 0.0 – 20.7) for WBC_2 above the median. The p-value was 0.261.
The total number of events was 5/10 in the WBC_2 below the median group, and 6/9 in the WBC_2 above the median group.
In the WBC_2 below the median, the OS after 12 months was 70.0% and the OS after 24 months was 50.0%. In the WBC_2 above the median group, the OS after 12 months was 33.3% and the OS after 24 months was 33.3%.

For ANC (absolute neutrophil count) three months after the initiation of treatment (ANC_2), the median was 7.0 10^9/L. Kaplan-Meier analysis of ANC_2 showed a median OS of 15.0 months (95% CI not applicable) for ANC_2 below the median and a median OS of 9.0 months (95% CI 0.0 – 20.7) for ANC_2 above the median. The p-value was 0.261.

The total number of events was 5/10 in the ANC_2 below the median group, and 6/9 in the ANC_2 above the median group.
In the ANC_2 below the median, the OS after 12 months was 70.0% and the OS after 24 months was 50.0%. In the ANC_2 above the median group, the OS after 12 months was 33.3% and the OS after 24 months was 33.3%.

For AEC (absolute eosinophil count) three months after the initiation of treatment (AEC_2), the median was 0.1 10^9/L. In a Kaplan-Meier analysis, AEC_2 below the median did not reach median OS, for AEC_2 above the median the median OS was 9.0 months (95% CI 0.2 – 17.8). The p-value was 0.109.
The total number of events was 4/10 in the AEC_2 below the median group, and 7/9 in the AEC_2 above the median group.
In the AEC_2 below the median, the OS after 12 months was 70.0% and the OS after 24 months was 60.0%. In the AEC_2 above the median group, the OS after 12 months was 33.3% and the OS after 24 months was 22.2%.

Parameter at Baseline *(N = 24, if not otherwise specified)*	**HR**	**95% CI**	**p-Value**
Sex	1.004	0.356 – 2.831	0.994
PD-L1 expression n = 18	0.991	0.973 – 1.009	0.326
Age	0.990	0.950 – 1.032	0.627
WBC	1.037	0.890 - 1.210	0.640
RBC	1.350	0.622 – 2.932	0.448
Hemoglobine	1.021	0.814 – 1.280	0.858
Hematocrite	1.012	0.927 - 1.104	0.793
MCV	0.866	0.749 – 1.002	0.052
MCH	0.753	0.539 – 1.054	0.099
MCHC	1.020	0.700 – 1.488	0.918
Platelets	1.000	0.996 – 1.004	1.000
MPV	1.034	0.605 – 1.769	0.902
% Neutrophils	1.008	0.954 – 1.065	0.777
Absolute Neutrophil Count	1.012	0.848 – 1.209	0.894
% Eosinophiles	1.148	0.904 – 1.459	0.258
Absolute Eosinophil Count	7.707	0.583 – 101.961	0.121
% Basophiles	0.743	0.263 – 2.099	0.575
Absolute Basophil Count	63.646	0.002 – 1685951,770	0.424
% Monocytes	0.991	0.811 – 1.210	0.928
Absolute Monocyte Count	0.958	0.245 – 3.740	0.951
% Lymphocytes	0.962	0.889 – 1.042	0.345
Absolute Lymphocyte Count	0.741	0.333 – 1.650	0.464
CRP n = 23	1.019	1.006 – 1.032	0.003
NLR	0.988	0.838 – 1.164	0.882

Table 6: Hazard ratios and 95% CIs calculated with univariate Cox regression of pre-treatment parameters for Pembrolizumab subgroup

Parameter at 3 months *(N = 19, if not otherwise specified)*	**HR**	**95% CI**	**p-Value**
WBC	1.205	1.007 - 1.443	0.042
RBC	0.774	0.312 – 1.919	0.581
Hemoglobine	0.763	0.530 – 1.099	0.146
Hematocrite	0.925	0.818 - 1.045	0.212
MCV	0.948	0.861 – 1.044	0.276
MCH	0.901	0.755 – 1.047	0.245
MCHC	0.871	0.620 – 1.225	0.428
Platelets	0.998	0.993 – 1.003	0.449
MPV	1.036	0.489 – 2.193	0.927
% Neutrophils	1.083	0.959 – 1.222	0.201
Absolute Neutrophil Count	1.287	1.028 – 1.611	0.028
% Eosinophiles	1.219	1.012 – 1.469	0.037
Absolute Eosinophil Count	2.906	1.086 – 7.774	0.034
% Basophiles	1.268	0.499 – 3.226	0.618
Absolute Basophil Count	45.764	0.002 – 1018407.489	0.454
% Monocytes	0.721	0.490 – 1.061	0.097
Absolute Monocyte Count	2.463	0.254 – 23.844	0.436
% Lymphocytes	0.919	0.822 – 1.028	0.139
Absolute Lymphocyte Count	0.624	0.204 – 1.909	0.409
CRP n = 18	1.030	1.004 – 1.057	0.025
NLR	1.126	0.992 – 1.279	0.067
$\Delta CRP_{1,2}$ n = 17	1.010	0.989 – 1.031	0.361

Table 7: Hazard ratios and 95% CIs calculated with univariate Cox regression of parameters three months after the initiation of treatment for Pembrolizumab subgroup

Parameter	HR	95% CI	p-Value
Age	0.956	0.887 – 1.031	0.241
CRP_1	1.030	1.006 – 1.055	0.015
WBC_2	0.823	0.134 – 5.072	0.834
ANC_2	1.215	0.132 - 11.178	0.864
AEC_2	5.338	0.439 – 64.852	0.189
CRP_2	1.094	1.023 – 1.170	0.009

Table 8: Hazard ratio's and 95% CIs from multivariable Cox regression analysis in Pembrolizumab subgroup

4 Discussion

4.1 Results and comparison to literature

This diploma thesis showed that pre-treatment and on-treatment CRP is a promising biomarker of response to immune checkpoint inhibitors in lung cancer.

CRP has been shown to be a promising biomarker of response to PD1/PD-L1 blockers in several types of cancer, including NSCLC. (100) The results from this thesis regarding pre-treatment CRP values are in line with existing research on the subject showing that elevated pre-treatment CRP is predictive of worse OS. The cut-offs that were determined using the median (14.0mg/L; 8.5 mg/L) are close to the currently most used cut-off in literature (10mg/dl). (100) In multivariable Cox regression, pre-treatment CRP was a stable predictor of OS in the Pembrolizumab subgroup, but not in the entire cohort. However, the hazard ratios of pre-treatment CRP in the entire cohort were the same in both univariate and multivariable analysis, suggesting that in the more heterogenous population the number of patients was too low to reach a significant result.

Elevated CRP three months after the initiation of treatment was associated with worse OS in the Pembrolizumab subgroup. Furthermore, the median CRP at three months was lower than at baseline (13.2 mg/L vs. 6.35 mg/L). Multivariable Cox regression analysis in this subgroup suggested that pre-treatment CRP and CRP at the three-month mark are independent predictive factors of OS. Riedl et al.'s study shows that a decrease in CRP during the first 8 weeks of treatment is associated with better progression-free survival, which is in line with some of the observations in this thesis. (94)

As described in the introduction of this thesis, the exact mechanisms as to why CRP predicts response to immune checkpoint inhibitors are unclear, but there is some evidence suggesting that CRP might suppress adaptive immunity in the TME. Furthermore, the tumour cells and cells in the TME actively stimulate CRP secretion by the liver, so a decrease in CRP after the initiation of treatment could possibly reflect response to treatment. However, it is unclear why on-treatment CRP independently predicts survival.

Even though some other parameters were significantly associated with OS in the univariate Cox regression analysis, none of these parameters remained stable predictors in the multivariable analysis. It is possible that our sample size was too small to pick up the smaller effects of these parameters. However, it is worth mentioning that the association of increased total neutrophils with worse OS was concordant with literature on this subject, as a decrease in neutrophils after treatment with ICIs has been associated with longer responses. (111) . For total leukocytes, there is no comparable data in literature, but since neutrophils make up a large proportion of leukocytes, it is possible the two could be correlated factors. Similarly, the association of on-treatment eosinophils with overall survival in the pembrolizumab subgroup could be because of the direct effects of ICIs on eosinophiles, but research on this is scarce and somewhat controversial, and mechanisms are unknown.

This data does not suggest that sex plays a role in the response to PD-1/L1 inhibitors in lung cancer. The sample size was too small to pick up on smaller effects of sex to response to ICIs, but there is no evidence for a difference between men and women in literature.

4.2 Limitations

The study has some limitations. The total number of patients in this study was low. It is possible that smaller effects were not picked up on due to low sample size. Considering that these biomarkers would ideally be used to predict outcome in an individual effects should be large. However, a larger sample size would have allowed us to further investigate the dynamic change in CRP during treatment by calculating the percentage decrease and to investigate the association between the trajectory of CRP and survival.

The study population was rather unselected, and we did not obtain information on treatment history. This is probably why better results were seen in the Pembrolizumab subgroup, as patients in this group were likely more homogenous, since Pembrolizumab is now a first line treatment for advanced NSCLC. Furthermore, no data on other potential influential factors on CRP, like smoking, cardiovascular disease, infections at time of measurement, use of other medications, and obesity was collected. Especially smoking history is known to have an influence on the efficacy of ICIs as well as CRP and should be included in future studies. Information on tumour size and stage also was not included in

the study, which is potentially problematic as CRP has been shown to be associated with tumour size in lung cancer.

Because of the small sample size, the median value was used as a cut-off in KM analysis to distinguish between high and low groups. To determine the ideal cut-off for lung cancer and specifically for the different ICIs and histological types of NSCLC, much larger studies will be necessary.

Another limitation of this thesis is that overall survival was the only endpoint used, and that it did not investigate progression-free survival, objective response rate, or quality of life, which are all important endpoints to consider when treating cancer.

A possible relationship between AEs and the investigated blood-based parameters. Since most AEs are immunomodulated and CRP, ANC and NLR are markers of inflammation, an increase in these parameters might be prognostic of the occurrence of AEs. Unfortunately, this is an often left out aspect in studies evaluating potential biomarkers of response to ICIs and should be investigated further in future research.

When it comes to the parameters measured after the initiation of treatment, additional points in time might be more useful for gathering information on treatment response. We only measured the parameters at routine visits in three-month intervals, while measuring it in shorter intervals could potentially be more useful in identifying patients at risk of being non-responders or at risk of developing AEs as fast as possible.

4.3 Implications of the results

If CRP was established as a biomarker of response, it would provide many benefits. CRP is a blood-based biomarker that is more readily available, cheaper, less invasive and associated with less complications and pain than tissue-based markers. Furthermore, it is likely to be influenced by all tumour lesions and therefore reflects an average of all the tumour sites, whereas tissue -based markers only assess a specific site. This also makes an ideal dynamic marker to be measured repeatedly over the course of treatment. (68)

Measuring CRP during treatment might be a simple way of gathering additional information on treatment response, apart from radiographic and clinical evidence, which is

especially valuable considering that more than two thirds of patients develop treatment resistance to anti-PD1/PD-L1 therapy. (115) Identifying non-responders to anti-PD1/PD-L1 therapy is essential for avoiding overtreatment, potential side effects and unnecessary costs, and most importantly allows providers to change the treatment regimen to a hopefully more successful option.

The development of inflammation markers as biomarkers of response to ICIs gives an insight into the function of tumour microenvironment and the interactions between the immune system and cancer that could even be of therapeutic use.
For lung cancer patients with high CRP, strategies to reduce inflammation and potentially alter the TME may be crucial in successful treatment with ICI and may prove a valuable anti-cancer strategy. CRP could possibly guide the development of treatment-enhancing combinations of ICIs with anti-inflammatory drugs, and examples of this are already being investigated. IL-1b and IL-6 receptor antibodies, which have originally been approved for several rheumatic diseases, have been shown to reduce immune-related adverse events of PD-1 blockers. Furthermore, the trial of the IL-1b antibody canakinumab showed a significant reduction in the incidence of lung cancer, and that patients with the greatest reduction in CRP and IL-6 reduced their risk the most. (102, 116) It has also been suggested that the inhibition of COX and therefore reduction of prostaglandin E2, which in turn reduces the release of IL-6 and CRP production, has a synergistic effect with PD-1 or PD-L1 blockers. (117, 118) Another potential option could be the use of statins since there is some evidence suggesting they lower CRP and enhance response to anti-PD1 therapy in NSCLC. (100, 119, 120)

The results of this thesis are meant to generate hypothesises. To validate these results and to establish standardized cut-offs, large and prospective studies will be necessary. Further research might also investigate the dynamic nature of CRP by modelling its trajectory. Furthermore, the impact of the genetic variants which influence baseline CRP production in a healthy individual could be investigated regarding their predictive value of response to immune checkpoint inhibitors. Even though the present thesis focuses on lung cancer, it is likely that the results will also provide valuable information for the development of biomarkers in other types of cancer.

In conclusion, this diploma thesis investigated a range of routine lab parameters as predictive markers in NSCLC treated with PD1/PD-L1 blockers in a real-world cohort and found an association of elevated pre- and on-treatment CRP with worse overall survival. If confirmed by additional studies, pre-treatment CRP and CRP after the initiation of treatment are easily measurable, minimally invasive as well as dynamic biomarkers that could contribute to the identification of patients who profit from checkpoint inhibitor therapy.

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Appendix

Grading of Adverse Events in Clinical Trials according to the Common Terminology Criteria for Adverse Events (CTCAE), Version 5.0, 2017:

Grade 1	Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.
Grade 2	Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental Activities of Daily Living (ADL).
Grade 3	Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self care ADL
Grade 4	Life-threatening consequences, urgent intervention indicated
Grade 5	Death related to AE

Table adapted from:
https://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/CTCAE_v5_Quick_Reference_8.5x11.pdf