UiT
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Immunological markers of non-small cell lung cancer

A retrospective tissue microarray study evaluating immune markers in the NSCLC tumor microenvironment, assessed by immunohistochemistry.

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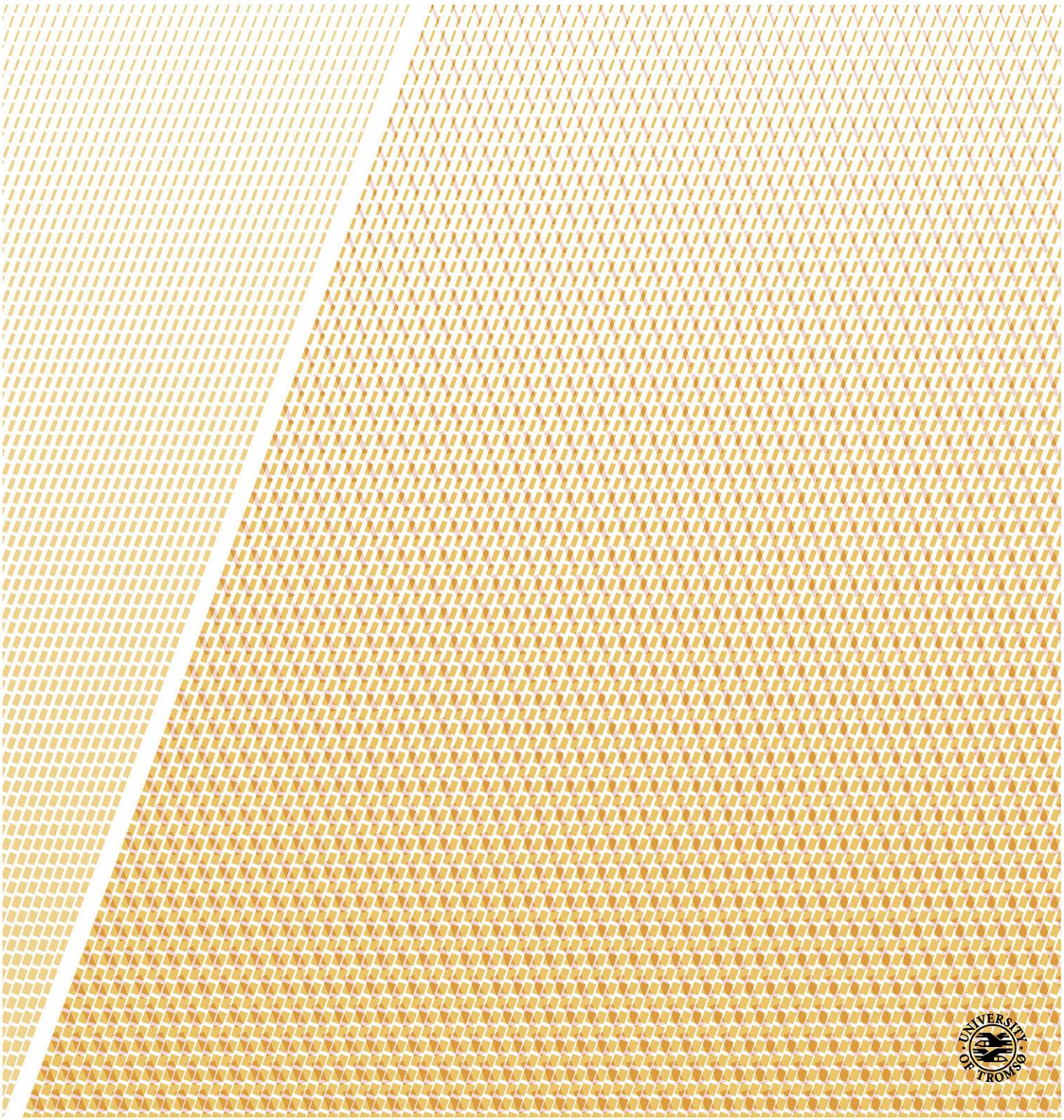

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PAPER II

Paulsen EE, Kilvaer TK, Khanehkenari MR, Al-Saad S, Hald SM, Andersen S, Richardsen E, Ness N, Busund, L-T, Bremnes RM, Donnem T. Assessing PD-L1 and PD-1 in Non-small cell ung cancer (NSCLC): a novel immunoscore approach. Clinical Lung Cancer 2016 (*In press, Accepted manuscript).* DOI:10.1016/j.cllc.2016.09.009

PAPER III

Paulsen EE, Kilvaer TK, Khanehkenari MR, Richardsen E, Hald SM, Andersen S, Busund L-T, Bremnes RM, Donnem T. CTLA-4 expression in the NSCLC tumor microenvironment: diverging prognostic impact in primary tumors and lymph node metastases. (*Submitted).*

LIST OF ABBREVIATIONS

ADC	Adenocarcinoma
AE	Adverse events
AIS	Adenocarcinoma *in situ*
ALK	Anaplastic lymphoma kinase
APC	Antigen-presenting cell
ASCO	American Society of Clinical Oncology
ATS	The American Thoracic Society
BAC	Bronchioloalveolar carcinoma
BRAF	A protein kinase encoded for by the proto-oncogene *B-Raf*
CAF	Carcinoma-associated fibroblast
CD	Cluster of differentiation
CK5/6	Cytokeratin 5/6
CRC	Colorectal cancer
CT	Central tumor
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
DAB	3,3'-Diaminobenzidine (DAB)
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DFS	Disease-free survival
DSS	Disease-specific survival
EBUS	Endobronchial ultrasound
ECM	Extracellular matrix
ECOG	Eastern Cooperative Oncology Group
EGFR	Epidermal growth factor
EMA	European Medicines Agency
ERS	The European Respiratory Society
ESMO	The European Society for Medical Oncology
EUS	Endoscopic ultrasound
FDA	The Food and Drug Administration, United States
FFPE	Formalin-fixed paraffin-embedded
FGF2	Fibroblast growth factor-2
FISH	Fluorescence *in situ* hybridization
Gy	Gray
H&E	Hematoxylin and eosin
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio
IASLC	International Association for the Study of Lung Cancer
ICC	Intraclass correlation
ICOS	Inducible T cell costimulatory
IDO	Indoleamine 2’3’ dioxygenase

IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IM	Invasive margin
KIR	Killer cell immunoglobulin-like receptor
LAG3	lymphocyte activation gene 3
LCC	Large cell carcinoma
LDCT	Low-dose computed tomography
LN	Lymph node
LN+	Metastatic lymph node
Mφ	Macrophage
M_1	M1 macrophage subset
M_2	M2 macrophage subset
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MIA	Minimally invasive adenocarcinoma
MET	A protein kinase encoded for by the proto-oncogene *MET*
N	Number
N+	Pathological nodal (N) stage 1-3
NA	Not assessed
NK	Natural killer cell
NH	Nordland Hospital
NOS	Not otherwise specified
NS	Did not stain
NSCLC	Non-small cell lung cancer
NTRK	A protein kinase encoded for by the proto-oncogene *NTRK1*
ORR	Overall response rate
OS	Overall survival
p (prefix)	Indicates pathological stage. E.g. pN2
PD-1	Programmed death-1 (receptor)
PD-L1	Programmed death-1 ligand
PDGF	Platelet-derived growth factor
PET	Positron emission tomography
PFS	Progression-free survival
PGE2	Prostaglandin E2
PT	Primary tumors
QoL	Quality of Life
RET	A protein kinase encoded for by the proto-oncogene *RET*
RCC	Renal cell carcinoma
ROS1	A protein kinase encoded for by the proto-oncogene *ROS1*
RNA	Ribonucleic acid
RR	Response rate
RT	Radiotherapy
S-	Stroma
SBRT	Stereotactic body radiation therapy

SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SLO	Secondary lymphoid organ
T-	Tumor
T790M	A specific mutation of the *EGFR* gene
T_{EM}	Effector memory T cell
T_H1	T helper 1 cell subset
T_H2	T helper 2 cell subset
T_{CM}	Central memory T cell
T_{reg}	T regulatory cells
T_{RM}	Tissue resident memory T cell
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Tim-3	T cell immunoglobin and mucin-3
TIL	Tumor infiltrating lymphocyte
TKI	Tyrosine kinase inhibitor
TLS	Tertiary lymphoid structure
TMA	Tissue microarray
TME	Tumor microenvironment
TNM	Tumor, noduli, metastasis, classification of pathological stage
TNF	Tumor necrosis factor
TTF-1	Thyroid transcription factor-1
UICC	International Union Against Cancer
UNN	University Hospital of North Norway
VEGF	Vascular endothelial growth factor
VISTA	V-domain Ig-containing suppressor of T cell activation
WHO	World Health Organization
WTS	Whole tissue sections

1 INTRODUCTION

Lung cancer is a major global health problem. Despite advances in prevention, diagnostics and therapies in the past decade, lung cancer is the leading cause of cancer-related morbidity and mortality worldwide[1]. Patients diagnosed with non-small cell lung cancer (NSCLC;~85% of lung cancers) are most often diagnosed at late stages, associated with dismal prognoses[2]. Patients who are diagnosed at early stages may undergo curative surgery, but many experience recurrences and eventually die from the disease. Hence, there is an urgent need to improve treatment strategies and survival for NSCLC patients.

The purpose of personalized medicine is to identify the optimal treatment for each individual patient, to maximize treatment benefit and minimize adverse effects. Informative biomarkers which can reliably predict outcome are needed to achieve this goal. In non-metastatic NSCLC and other solid cancers, staging according to the TNM system has been the most important clinicopathological variable for prognostication and stratification of patients, and an essential guide to therapy-related decisions in clinical routine[3]. However, the clinical outcome of patients classified with the same pathological (TNM) stage disease differ considerably, and there is a lack of other validated biomarkers. Identifying novel variables that characterize patients likely to have poor outcomes may help direct clinicians to personalize treatment for patients, and may also aid in research for new therapeutic options.

Our knowledge of cancer genes and mutational processes, and their evolution during tumor development has led to an increased understanding of the genetic heterogeneity among cancer cells. and targeted therapy has offered new hope to NSCLC patients. Moreover, it has become evident that cancers develop in complex tissue environments. Malignant cells interact closely with their neighboring non-malignant stromal cells in a complex and dynamic tumor microenvironment (TME). Inflammation is a hallmark of cancer, and most adult solid tumors contain infiltrates of diverse immune subsets which can kill or suppress cancer cells or be co-opted by the tumor and support cancer progression and metastasis[4]. An improved understanding of the interactions between cancer cells and the immune system has prompted development of new drugs that therapeutically exploit the body's immune system to fight the cancer.

However, like cancer cells, tumor immune microenvironments are heterogeneous. It has been demonstrated that the balance between pro- and antitumor immune factors in the TME defines whether a tumor will be eliminated by, survive in equilibrium with, or escape the immune system[5]. Deciphering which immune cell subsets, and mechanisms regulating the function and activity of these, control the outcome of cancer, is presently under intense scrutiny. Furthermore, the development of reliable immunological criteria, which can supplement current tumor-autonomous prognostic factors, may enable clinicians to more precisely identify patients at high and low risk, and select patients for treatments and thereby improve patient outcomes.

1.1 Lung Cancer

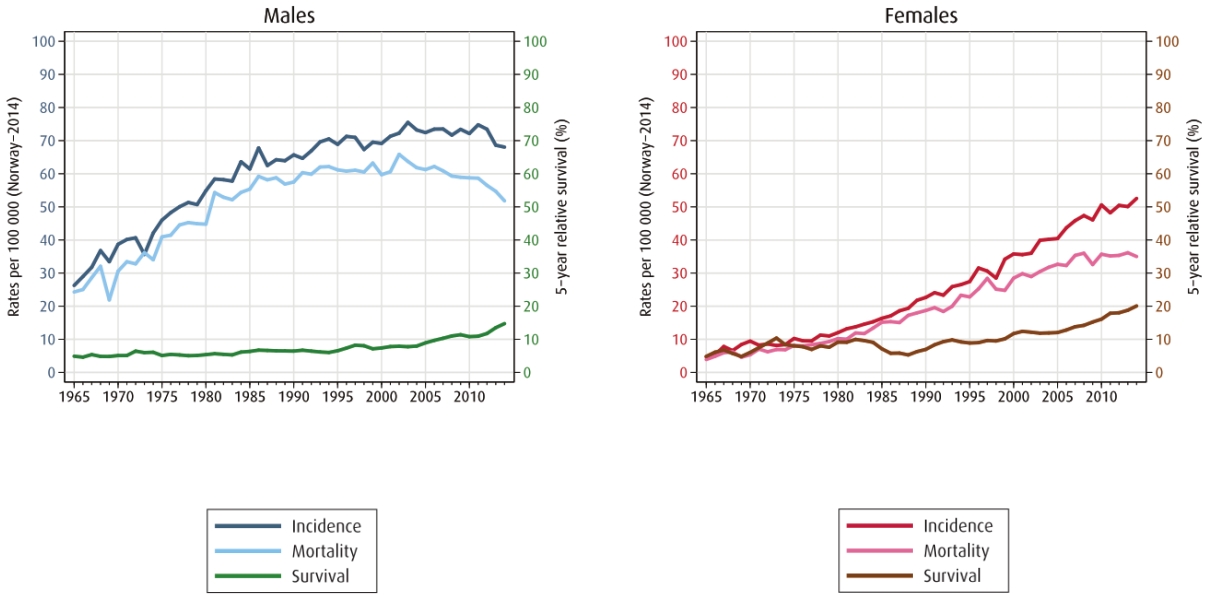
1.1.1 Epidemiology

Lung cancer remains the most frequently occurring cancer worldwide, with approximately 1.8 million new cases annually[1]. Sadly, it is also one of the most aggressive human cancers, with an estimated 1.6 million deaths in 2012; incidence and mortality rates follow each other closely[1].

In males, lung cancer is both the most frequently diagnosed cancer and the leading cause of cancer death worldwide, while in developed countries, including Norway, prostate cancer is more commonly diagnosed[1,6]. In females, breast and colon cancer are more commonly diagnosed worldwide, but lung cancer is the leading cause of cancer death in more developed countries, and second only to breast cancer in less developed countries[1,6].

In Norway, there were 3019 new cases of lung cancer diagnosed and 2158 lung cancer deaths in 2014. In fact, lung cancer represented one in five cancer deaths[6] (Figure 1). In 2012, lung cancer caused almost as many years of life lost alone, as colon, prostate and breast cancer combined[7].

Figure 1: Trends in incidence and mortality rates and 5-year relative survival proportions (adapted from www.kreftregisteret.no; Cancer in Norway 2014)



The majority of patients are diagnosed with advanced stage disease (70-80% stage IIIB-IV), and only 20-30% of patients are candidates for surgical resection (stage I-IIIA), contributing to a poor 5-

year overall survival rate of around 15% for all stages combined[2]. According to the most recent international TNM registration study by IASLC (International Association for the Study of Lung Cancer), the 5-year NSCLC survival declines gradually from Stage IA (82%) to stage IV (6%) (Table 1; see 1.1.2)[3]. There was an overall improved survival compared to the last IASLC TNM registration study (2007), and reasons for this were considered to be improved diagnosis (increased LDCT screening, PET, endobronchial ultrasound (EBUS) and endoscopic ultrasound (EUS)) and treatment (increased use of adjuvant therapy, stereotactic body radiation therapy (SBRT) and minimally invasive surgery for treating less fit individuals, novel targeted agents for stage IV disease)[3].

The major cause of lung cancer is tobacco smoking, estimated to account for approximately 80-90% of lung cancer cases in high-income countries in 2014[8]. Incidence rates of lung cancer have risen dramatically since the mid-20th century, reflecting the evolution of the smoking epidemic[9]. Secondary to a decline in smoking prevalence, lung cancer incidence for men in high-income countries began to level off in the 1990s and are gradually declining[10]. Since women took up smoking in large numbers later than men, at older ages, and were slower to quit, lung cancer incidence has continued to rise in women in most countries[11].

In addition to exposure to tobacco smoke through active or passive smoking, other known risk factors for lung cancer include occupational or environmental exposure to asbestos, nickel, chromium and arsenic, radiation including radon gas, smoke from cooking and heating, and outdoor air pollution[12].

Traditionally, the predominant lung cancer histology has been squamous cell carcinoma (SCC) in men and adenocarcinoma (ADC) in women and non-smokers. Smoking is known to exert a steeper risk gradient on SCC than ADC, and in recent years, the rates of SCC in males have decreased while ADC rates have been increasing in both females and males[13,14]. ADC is now the most frequent histological group in men and women in Norway, and the percentage of nonsmokers, particularly with ADC histology is growing[9,12]. The increase in ADC is considered to be related to modifications in the tar and nicotine content of cigarettes, and the introduction of filters which may have led to deeper inhalation of small particles into distal airways[14].

1.1.2 Histopathology

Lung cancer is classified into two major groups, small cell lung cancer (SCLC; 15%) and non-small cell lung cancer (NSCLC; 85%)[15]. Historically, NSCLC tumors have been defined primarily by the use of light microscopy and morphological subtyping, and NSCLC tumors were lumped together because dividing them into subtypes had no therapeutic implication[16]. In recent years, advances in lung cancer genetics and treatment have demonstrated that NSCLC is a heterogeneous entity and major changes for the pathological classification of tumors have been introduced[17]. Pathologists now play an important role in personalized medicine for lung cancer patients, as treatment decisions are heavily dependent on histologic subtype and molecular characteristics of the tumor.

Until recently, the three most common histological types of NSCLC were adenocarcinoma (ADC), squamous cell carcinoma (SCC) and large cell carcinoma (LCC), each with distinguished morphological features. The 2011 lung adenocarcinoma classification, further adopted into the 2015 WHO Classification of Lung Tumors, introduces efforts to perform increasing refinement of pathological diagnosis on the basis of more expanded use of immunohistochemistry (IHC) and genetic testing, and defines terminology and criteria to be used in small biopsies and cytology[2,18]. The IHC analyses to further classify tumors include adenocarcinoma markers (i.e. TTF-1) or squamous markers (i.e. p40 or p63, CK5/6) and/or mucin stains, leaving NSCLC-NOS (not otherwise specified) to be used as little as possible[18]. With the introduction of special stains, the entity LCC has become increasingly rare and instead reclassified as SCC, ADC or NOS[18].

The current recommendations include the discontinuation of the use of the term bronchioloalveolar carcinoma (BAC), which has been reclassified as 1) invasive adenocarcinoma (sub-classified by predominant pattern); 2) minimally invasive adenocarcinoma (MIA) and 3) adenocarcinoma *in situ* (AIS), as the two former have excellent prognoses when undergoing complete resection[2].

For patients with advanced-stage disease, the distinction between ADC and SCC has become imperative in order to guide treatment strategies and predict clinical course, for several reasons[17]. In terms of therapy, pemetrexed (chemotherapy) has little or no activity in SCC, and bevacizumab (angiongenesis inhibitor) has excessive toxic effects in SCC[19]. Recent discoveries that specific molecular pathways drive cancer progression have made molecular testing for mutational status, particularly in adenocarcinomas, increasingly important[2,18].

Due to the availability of targeted therapy, all Norwegian patients with non-SCC NSCLC are tested for epidermal growth factor (EGFR) mutations (DNA-based) and anaplastic lymphoma kinase (ALK) rearrangements (IHC, supplemented by FISH) at the time of diagnosis. Activating mutations in the EGFR gene are found in ~10-16% of Caucasians with adenocarcinoma and ~50% in Asian countries, and are more frequent in never smokers and females[19,20]. ALK fusions are found in ~3-5% of patients with adenocarcinoma subtype, predominantly in non-smokers and younger patients[19]. Other genetic aberrations less frequently tested for, which may allow access to targeted treatment in late lines of therapy, include RET, ROS1, HER2, BRAF, MET and NTRK[21].

1.1.3 Diagnosis, staging (TNM) and prognosis

The main symptoms associated with a lung cancer diagnosis such as fatigue, persistent cough, weight loss, breathlessness and chest pain, are also associated with age and a smoking history, thus might be misinterpreted and contribute to a delayed diagnosis[20]. Reduced appetite, weight loss and fatigue is associated with more advanced stages of disease[20]. Asymptomatic tumors may also be discovered by incidental detection.

Early diagnosis and treatment may reduce lung cancer mortality, but early screening programs for lung cancer involving chest radiographs and sputum cytology did not lead to reduced lung cancer mortality[22]. In 2011, annual low-dose computed tomography (LDCT) screening of specific high-risk groups (≥30 pack-years) was documented to significantly reduce lung cancer mortality in the American National Lung Screening Trial, however, optimal methods of defining the population and screening interval to avoid over-diagnosis and over-treatment are not clear[22,24]. American health authorities have opened for LDCT screening for a selected high risk population, and countries such as Canada and China have included recommendations in national guidelines[21]. The European Society for Medical Oncology (ESMO) recommended in 2014 LDCT screening to be carried out only within a dedicated program at selected, high-volume centers of thoracic oncology expertise, accompanied by individual smoking cessation counseling[22]. More recent studies favor LDCT screening, but further research to improve screening efficiency is ongoing[21]. In Norway, recommendations from a working group assessing lung cancer screening implementation is expected by the end of this year[20].

For patients with suspected lung cancer, it is imperative to ensure a timely diagnosis and accurate staging, so that the appropriate therapy may be initiated without delay[20]. Patients with a

clinical suspicion of lung cancer must, upon liberal indication, immediately be referred to radiologic imaging for clinical staging[20]. An initial chest X-ray is often initially performed, supplemented or replaced by a chest computed tomography (CT) including the upper abdomen, liver and adrenal glands. This should ensure the identification of a potential primary lung tumor, regional and distant metastases in the liver and adrenal glands, and an optimal tissue sampling[20]. Tissue for histopathologic examination is acquired by biopsy or cytology, preferably via bronchoscopy, EBUS or EUS, or CT-guided biopsy if tumors are peripheral[20]. Obtaining adequate tissue material for histological diagnosis and molecular testing is important. PET-CT, MRI of the brain, thorax or spine/pelvis and mediastinoscopy are modalities used to achieve a final clinical staging.

Lung cancer tumors are currently staged according to the IASLC 7th edition of the TNM (tumor, noduli, metastasis) classification (Table 1), which was validated by the analysis of more than 67,000 cases of NSCLC treated using all modalities of care between 1990 and 2000, and was implemented in January 2010[25]. It is presently the single most important prognostic guide for treatment allocation of NSCLC patients, such as the use of adjuvant therapy.

A revision of the TNM classification will be implemented in 2017[3]. The most recent IASLC database contains more than 94,000 cases, treated by all modalities of care between 1999 and 2010, of which 85% went through surgery[3]. Proposed changes to some T and M descriptors will result in some cases being assigned to a different stage in the 8th than they would have been in the 7th edition[3]. Tumor size is emphasized, and will be a descriptor in all T categories, a new stage IIIC (T3/T4 N3 M0) and IVB (Any T, Any N, multiple metastases) will be introduced[3]. A sharper distinction between subsets of T, N and M categories and stage groups are accomplished, hopefully of clinical relevance. However, whether these changes will add to the effectiveness of treatment for NSCLC remains to be assessed in appropriate clinical trials[3].

Table 1 The 7th edition of TNM classification and stage groupings (Table adapted from[25])

Stage	Sub-stage	T Category	N Category	M Category	5-year OS IASCL 2007[26]	5-year OS IASCL 2016[27]
Occult carcinoma		TX Primary tumor not assessed , or proven only by cells or imaging	N0 No regional lymph node metastases	M0 No distant metastasis		
Stage 0		Tis Carcinoma *in situ*	N0			
Stage I	**IA**	T1a Tumor ≤2 cm	N0		73%	83%
		T1b Tumor ≤ 3cm <2 cm				
	IB	T2a Tumor ≤ 5cm <3 cm[a]	N0		58%	71%
Stage II	**IIA**	T1a	N1 Metastasis in ipsilateral peribronchial/hilar/intrapulmonary LN		46%	57%
		T1b				
		T2a				
		T2b Tumor ≤ 7 cm <5 cm[a]	N0			
	IIB	T2b	N1		36%	49%
		T3 Tumor > 7cm[b]	N0			
Stage III	**IIIA**	T1	N2 Metastasis in ipsilateral mediastinal and/or subcarinal LN		24%	36%
		T2				
		T3	N1			
		T3	N2			
		T4 Tumor invading mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, oesophagus, vertebral body, or tumor in different ipsilateral lobe	N0			
		T4	N1			
	IIIB	T4	N2		9%	23%
		Any T	N3 Metastasis in contralateral mediastinal/hilar LN or scalene/supraclavicular LN			
Stage IV	**IV**	Any T	Any N	M1a	13%	

[a]:With any of the following features: involves main bronchus, ≥2 cm distal to the carina, invades visceral pleura, atelectasis/obstructive < the entire lung.[b]:Or that directly invades any of the following: parietal pleura, chest wall, diaphragm, phrenic nerve, mediastinal pleura, parietal pericardium or tumor in the main bronchus (<2 cm distal to the carina, atelectasis/obstructive pneumonitis of the entire lung). Abbreviations: LN: lymph node.

1.1.4 Treatment of NSCLC

Surgery, chemotherapy, radiotherapy, or combinations of these, are the most important treatment modalities for NSCLC patients. However, SBRT, targeted agents, and the recent development in immunotherapy have improved outcomes for patients with locally advanced or advanced stage disease. Although conventional chemotherapy generally targets replication strategies in tumor cells, preclinical evidence suggests that the effect may also occur through modulation of the immune system, e.g. by the triggering of immunogenic cell death, uptake and processing of tumor antigens, and depletion of immunosuppressive cells[28].

1.1.4.1 Curable NSCLC

If there are no contraindications, patients with stage I-III disease may be treated with curative intent, but have a significant risk of recurrence and death. Complete surgical resection is the preferred option for stage I disease patients who are surgical candidates, increasing overall survival rate from 6% for non-operated patients, to 55-77% for patients treated with lobectomy[29].

For stage II and IIIA patients, surgery and adjuvant therapy is the recommended treatment[20]. If comorbidity or patients' preferences precludes surgery, definitive radiotherapy (RT) is an option for stage I-III NSCLC patients[20]. Conventional RT has moderate side-effects and can increase 3-year survival from negligible to 32% (stage I/II)[30]. Today, SBRT is a favorable option for medically inoperable stage I and T2bN0-disease[20]. The technique supplies a high radiation dose to tumor tissue, while largely sparing normal tissue. Few treatments (3-5) are delivered at high doses, and studies show results comparable to surgery[31,32].

The adjuvant chemotherapy regimen recommended for NSCLC in Norway is four cycles of cisplatin/ vinorelbine, offered to stage II and IIIA patients ≤ 70 years of (biological) age and with acceptable performance status (ECOG 0-1)[33,34]. Due to adverse events, not all patients complete all four cycles[20]. Neoadjuvant chemotherapy is not recommended outside of clinical studies, as improvement in survival does not outcompete that of adjuvant therapy[20].

Stage III patients are a heterogeneous group in which defined prognostic factors (performance status and weight loss) are considered with T and N stage, before choice of therapy is decided within a multidisciplinary approach[20]. For stage IIIA patients, concomitant chemotherapy and RT is recommended, but sequential therapy, combinations with surgery or RT alone may also be

considered[20]. Patients with stage IIIB disease are not curable by surgery, but concomitant or sequential chemotherapy and RT should be considered[20].

In cases where pN2 lymph nodes are discovered perioperatively, patients should be offered postoperative RT (50-54 Gy) after attempted complete ipsilateral lymph node dissection[20]. Patients with positive surgical margins are also treated with postoperative RT, whenever re-resection is not possible (60-70 Gy) [20].

In Norway, the first recommended control after curatively intended treatment is at 4-6 weeks (surgery: X-ray at 4 weeks, RT: computer tomography at 6 weeks), followed by clinical examination and computer tomography at 6,12,18 and 24 months and 3, 4 and 5 years[20].

1.1.4.2 Advanced NSCLC

The majority of NSCLC patients present with advanced stages (40% stage IV, 30% earlier stage disease, but with negative prognostic factors), and treatment options are further limited by smoking- and age-related comorbidities[35]. Unfortunately, the quality of life (QoL) of lung cancer patients is worse than for other cancers[29]. Hence, early palliative/supportive care integrated with standard oncologic care is imperative, and may significantly improve QoL[36]. In any stage of NSCLC, smoking cessation improves outcome[37]. To avoid over-treatment, it is important to assess performance status and weight loss, and to be aware that palliative chemotherapy is associated with significant toxicity. However, systemic therapy for advanced NSCLC patients may delay disease progression, prolong survival and improve QoL[37]. Directed RT should be used to alleviate QoL-reducing symptoms from tumors/metastases, commonly in central airways, skeleton or brain[20].

In patients with non-SCC tumors, EGFR-mutations are predictive for response to the EGFR tyrosine kinase inhibitors (TKI), erlotinib, afatinib, gefitinib, which show improved response rates (RR), progression-free survival (PFS) and QoL compared to chemotherapy in the first line setting[37]. The dual ALK- and MET-kinase inhibitor crizotinib is recommended for patients with ALK-rearrangements in first line, and ceritinib in second line, but monitoring with regard to side-effects is important. Recently, the third generation EGFR-inhibitor osimertinib has been approved by FDA and EMA for patients with T790M-mutation after previous treatment with an EGFR-TKI. Chemotherapy is indicated upon progression on TKI treatment[20].

For patients with SCC histology, or whose tumor does not contain a driver mutation for which a targeted agent is available, cytotoxic chemotherapy is usually the initial therapy. Platinum-based (carboplatin or cisplatin) double alternatives with vinorelbine, gemcitabine, paclitaxel, docetaxel or pemetrexed (only non-SCC) are considered equal, and 3-4 cycles are recommended for patients with ECOG 0-2[20]. In Norway, 3-4 cycles of the carboplatin/vinorelbin doublet is recommended due to toxicity, QoL and cost-efficiency profiles[20]. Compared to BSC (best supportive care) the absolute survival benefit after 1 year is around 8% and improved QoL[20,37]. Maintenance therapy with pemetrexed for patients with non-SCC histology may be considered after 3-4 cycles of platin-doublet chemotherapy[20].

Second line single-agent chemotherapy, pemetrexed (non-SCC) or docetaxel, should be recommended to patients with ECOG 0-1 who progress on first line chemotherapy[20]. Docetaxel has an overall response rate (ORR) of <10%, median PFS around 2-3 months and median OS around 7 months[38]. EGFR-TKI, ramucirumab (vascular endothelial growth factor receptor, VEGFR-2-inhibitor) or nintedanib (angiokinase inhibitor, ADC only) with docetaxel are other treatment options recommended by ESMO[20].

Immunotherapy has become a promising new approach for NSCLC patients. “Releasing the brakes” of the anti-tumor immune system, by blocking inhibitory signals or triggering co-stimulatory signals to amplify tumor antigen-specific T cell responses, is currently the subject of intense study in cancer, including NSCLC[39]. Drugs blocking immune checkpoint receptors CTLA-4 (cytotoxic T-lymphocyte-associated antigen 4) with monoclonal antibodies (ipilimumab/tremelimumab) were the first immune checkpoint inhibitors explored in clinical trials for NSCLC patients[39]. CTLA-4 blockade has been associated with increased toxicity and less therapeutic efficacy compared to drugs targeting the Programmed death-1 (PD-1) pathway in NSCLC patients, but the combination has yielded the most impressive results[40].

In 2015, two new immunotherapeutic agents were approved by the FDA and EMA for the treatment of locally advanced or metastatic NSCLC patients in the 2nd line setting; nivolumab and pembrolizumab, both PD-1 inhibitors. Nivolumab was initially approved for patients with SCC histology after progression on platinum-based therapy, based on the CheckMate 017 study[41]. Compared with the docetaxel-arm, improvements were seen in median OS (9.2 vs 6.0 months), ORR (20% vs 9%) and 18 month OS (28% vs 13%)[41]. It was better tolerated than docetaxel (Grade 3-4

adverse events (AE): 10% vs 54%) and showed a positive impact on QoL[37]. Of note, the expression of PD-1 ligand (PD-L1) was neither prognostic nor predictive of clinical benefit.

Shortly after, nivolumab was also approved for non-SCC patients. The CheckMate 057 trial showed a similar benefit in OS, RR and AE, except for never-smokers and EGFR-mutated subgroups[42]. A retrospective analysis demonstrated an association between tumor membrane PD-L1 levels (≥1%, ≥5%, ≥10% tumor-membrane positivity) and treatment efficacy of nivolumab. In the PD-L1 negative group nivolumab efficacy was comparable to that of docetaxel, but with less AEs[42].

In the Keynote-010 study (pembrolizumab vs. docetaxel, 2nd line advanced), only PD-L1 positive patients (≥1%) were included, independent of histology[43]. PD-L1 expression levels were predictive of effect, and with an optimal cutoff point of ≥50%, ORR was 58% and 24 month OS 61%[43]. Similar tolerance and lack of effect in EGFR-mut+ patients was seen, as was for nivolumab[43]. PD-L1 inhibition has also shown benefit over docetaxel in NSCLC, and early studies show promising results of both PD-1 and PD-L1 inhibition in the 1st line setting, and post-surgery trials are ongoing[44–48].

The selection of patients to programmed death 1 (PD-1) pathway blockade by PD-L1 positivity is controversial because of a suboptimal negative prognostic value[49]. Two commercial PD-L1 IHC assays are presently available. The Dako PD-L1 IHC 223C pharmDx is a companion diagnostic required for treatment with pembrolizumab (≥50% Tumor Proportion Score necessary) while the Dako PD-L1 IHC 28-8 pharmDx is a standalone complimentary diagnostic test (≥1%, ≥5%, ≥10% tumor-membrane positivity, approved for non-SCC NSCLC).

ESMO recommendations for 2nd line treatment of EGFR- and ALK-negative NSCLC (SCC and non-SCC) includes both nivolumab, and pembrolizumab (in PD-L1+ patients only, as determined by a companion diagnostic assay)[37]. Norwegian health authorities have recently (Sept, 2016) granted access to the public for pembrolizumab treatment only, in this setting, after a national cost-benefit assessment[50]. This requirement for testing is a challenge for the many Norwegian pathology departments, which lack established technique and/or appropriate equipment and training.

1.2 The immune system and cancer

1.2.1 The tumor microenvironment

In normal adult tissues, stromal cells closely interact to maintain tissue homeostasis and prevent tumor formation. In cancer, malignant cells can reside in, transform and eventually recruit the adjacent stroma to support tumor growth and facilitate metastatic dissemination[4]. The biology of the stromal compartment in cancer involves a balance between tumor-promoting and tumor-inhibiting mechanisms.

The TME consists of a complex milieu of extracellular matrix (ECM), fibroblasts and vascular cells, infiltrating immune cells and soluble factors such as cytokines and chemokines[51,52]. Tumor cells can modulate their stromal environment by secreting signal molecules such as growth factors and proteases, which can act in autocrine and paracrine manners, or by cell-to-cell interaction[4]. Activated stromal cells contribute to the cancer cell-permissive environment by the release of growth factors, cytokines, and chemokines[53]. Proteases contribute to the degradation of the basement membrane and ECM, releasing tumor-promoting cleavage products from ECM components[4]. Driven by growth factors such as transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), fibroblast growth factor-2 (FGF2), fibroblasts can differentiate into a tumor promoting and diverse set of cells termed carcinoma-associated fibroblasts (CAFs). CAFs produce growth factors and ECM, and contribute to recruitment and activation of immune cells and epithelial-mesenchymal transition[54–56]. Angiogenic factors such as VEGF (vascular endothelial growth factor) are mainly produced in CAFs and inflammatory cells, and contribute to the formation of a high number of newly formed leaky tumor blood and lymph vessels [56,57].

Since most adult solid tumors contain infiltrates of inflammatory cell subsets, wherein immune cells exert either pro- or anti-tumor properties, inflammation has been recognized as a hallmark of cancer[58,59]. The composition and activation status of these diverse myeloid and lymphoid-lineage subsets vary greatly depending on “host” tissue and stage of the malignant disease. Tumors develop in a complex and dynamic interaction with the immune system, both the innate and adaptive, through processes collectively termed immunoediting[5,60].

One of the most important aspects of the tumor-microenvironment crosstalk is how cancer cells modulate and interfere with the inflammatory response, e.g. by altering the T cell response from the T helper 1 (T_H1) cell subset to the T_H2 cell subset, the induction of immunosuppressive T

regulatory (T_{reg}) cells, a skewing of the phenotype of macrophages and neutrophils to a type 2 differentiation state, and the induction of myeloid-derived suppressor cells (MDSCs)[61].

Finally, it has been shown that the immune contexture, defined as the type, location, density and functional orientation of the different immune cell populations, affects the prognoses of cancer patients[62].

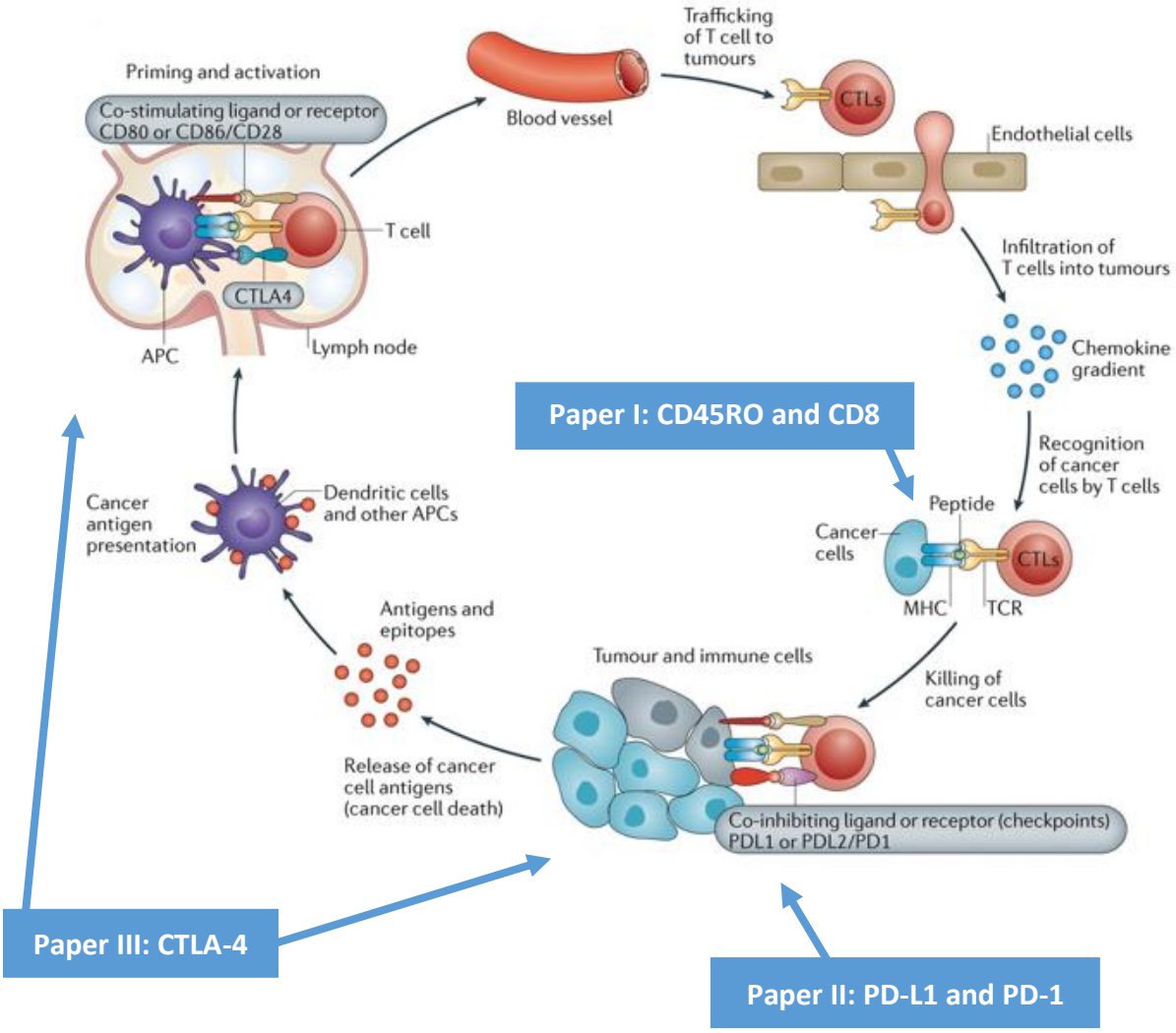
1.2.2 The Cancer-Immunity Cycle: Roles of innate and adaptive immunity

A series of stepwise events must proceed in order for the anticancer immune response to mediate effective killing of cancer cells, termed the Cancer-Immunity Cycle by Chen et al[63]. These steps include the capture and presentation of tumor antigens, by antigen-presenting cells (APCs), to T cells. The activated effector T cells traffic to the tumor bed, where they recognize and kill their target cancer cell. The killing of tumor cells releases additional tumor antigens, and the cycle is re-initiated as illustrated in Figure 2.

APCs (dendritic cells (DCs) and macrophages (Mφs)) are responsible for identifying, capturing and processing exogenous proteins, and present antigen peptides to T cells. In order for them to be recognized by T cells, antigens must be presented in the context of major histocompatibility complex (MHC) molecules. Although such T cell "priming" is thought to primarily occur in tumor-draining lymph nodes, studies suggest that T cell education can also occur in the tumor stroma, such as in spontaneously organized tertiary lymphoid structures (TLSs)[61]. The presence of TLSs is seen in the tumor stroma in some cancers, comprising a T cell zone with mature DC adjacent to a B-cell follicle including a germinal center, surrounded by high endothelial venules, potentially contributing to the activation and education of naïve T cells into memory effector T cells[37].

Tumor-reactive T cells can potentially recognize, and subsequently reject, cancer cells which express neoantigens (novel protein sequences formed as a consequence of somatic mutations and loss of normal cellular regulatory processes) and non-mutated self-antigens which are overexpressed, or to which T cell tolerance is incomplete [61].

Figure 2: The Cancer Immunity Cycle. Cancer cell antigens are released, captured by dendritic cells and other APCs and presented on MHC molecules to T cells, resulting in the activation of effector T cell responses against the cancer-specific antigens. Guided by a chemokine gradient, activated T cells traffic to and infiltrate the tumor site, where they recognize and bind to cancer cells via the TCR, and cytotoxic T lymphocytes kill the cancer cells. New cancer antigens are released and the T cell response may be broadened. Co-stimulatory and co-inhibitory checkpoints regulate T cell activation in secondary lymphoid organs (SLOs) and within cancer tissues, as a result of tumors co-opting the physiological immune regulatory feedback and tolerance mechanisms. As published in[68], adapted from[63]. Permission obtained from Nature Publishing Group©.



DCs are extremely efficient at activating antigen-specific T cells[66]. DCs are triggered by danger-associated molecular patterns (DAMPs) from injured host cells or inflammatory mediators such as TNFα, IL-1β, IL-6 or PGE2, and may encounter tumor antigen at the tumor site or soluble antigen transported to lymph nodes (LN) via lymphatic vessels[67]. They use numerous pattern recognition receptors (e.g. Toll-like receptors) to detect tumor antigen, and present it to T cells via MHC molecules. This represents a critical link between the innate and adaptive immune system, because T cells cannot recognize unprocessed antigens[66]. DCs also secrete immune modulatory cytokines which can further support or inhibit the anti-tumor response[67].

When antigen-naïve CD4+ and CD8+ T cells encounter a foreign (non-self) antigen bound to an MHC molecule (pMHC) through their T cell receptor (TCR), they receive a "first signal" to mount a response specific to that antigen. But this requires the appropriate help from cytokines and co-stimulatory molecules ("second signal") (Chapter 1.2.3). Activated T cells differentiate into effector T cells, expand in numbers, traffic to and infiltrate the tumor site, where they may recognize cancer cells through the interaction between its TCR and pMHC on cancer cells. They kill their target cancer cell, releasing additional tumor antigens which can increase the width and depth of the response[63].

CD8+ T cells are considered the major anticancer effector cells, as they can differentiate into cytotoxic T lymphocytes (CTL) in the context of co-stimulatory signals and APC-derived cytokines (such as IL-12, tye 1 IFN and IL-15)[69]. CD8+ T cells can mediate killing of cancer cells through mechanisms such as the release of cytotoxic mediators (granzyme A and B, perforin), the secretion of cytokines such as IFNγ and TNFα which can promote Mφ cytotoxic activity, and the activation of apoptotic pathways[69].

Naïve CD4+ T cells can give rise to helper cells with distinct cytokine profiles, which orchestrate diverse immune responses. Th1-polarized CD4+ T cells assisting CD8+ T cells in suppressing tumors by secreting IL-2, TNFα and IFNγ, and promote Mφ cytotoxic activity and expression of MHC on APCs[4].

1.2.3 Co-stimulatory and co-inhibitory signals

For a naïve T cell, stimulation through the TCR alone is not sufficient for activation. Co-stimulatory signals, typically mediated by ligands expressed on APCs ("second signal"), are required to regulate the amplitude and quality of the response[70]. The most recognized co-stimulatory molecule, CD28, is expressed on T cells and interacts with B7-1 (CD80) and B7-2 (CD86) on APCs, stimulating T cell proliferation, cytokine production, and survival[39,71]. Other co-stimulatory molecules which regulate T cell responses are CD137 (4-1BB), OX40 and ICOS (inducible T cell costimulator)[70,72].

Similarly, co-inhibitory signals, termed checkpoints, negatively modulate the activation and differentiation of the T cell, mediated by T cell receptors such as CTLA-4, PD-1, LAG3 (lymphocyte activation gene 3) and Tim-3 (T cell immunoglobin and mucin-3)[39]. KIR (killer cell immunoglobulin-like receptor) ligation inhibits NK-cell function, VISTA (V-domain Ig-containing suppressor of T cell activation) is expressed mainly on myeloid cells and IDO (indoleamine 2'3' dioxygenase) inhibits T cells locally via conversion of tryptophan, essential for T cell survival and effector function[39]. The balance between co-stimulatory and inhibitory signals is crucial for the maintenance of self-tolerance under normal physiological condition. The normal mechanisms which prevent autoimmunity and collateral damage to normal tissues in the course of the immune response to pathogens can be co-opted by cancer to evade immune destruction.

1.2.4 Development of T cell memory

Following the clearance of antigen and resolution of inflammation, pro-inflammatory cytokine levels subside, immunosuppressive T_{reg} numbers increase and DCs are suppressed. Most activated T cells die during this contraction phase, but a subset of T cells transition into long term survivors. These cells can mature into memory T cells and can provide long-term immunity with rapid reactivation of effector function upon antigen re-encounter[73].

Memory T cells are diverse with regard to localization and functions, and can typically be divided into 1) central memory cells (T_{CM}), which reside in SLOs and can rapidly expand and differentiate upon re-exposure to the antigen; 2) effector memory cells (T_{EM}), which are capable of immediate cytotoxicity and traffic the circulation and mucosal sites; and 3) tissue-resident memory T cells (T_{RM})[74]. Of note, memory is developed after antigen stimulation and inflammation has resided,

and require low levels of pro-inflammatory signals. In cancer, persistent antigen stimulation and chronic inflammation ensues, and can alter memory T cell differentiation[73].

1.2.5 The three E's of immunoediting

Early studies by Schreiber et al. revealed that the immune system could recognize and reject malignant cells (immunosurveillance), and studies performed in the last decade have established that the immune system further shapes the character of emerging tumors (immunoediting), in three phases – Elimination, Equilibrium and Escape[5,75–77] (Figure 3).

According to the immunoediting theory, the host can control tumor growth through the activation of adaptive and innate immune mechanisms during the elimination phase. Driven by host anti-tumor responses, including presentation of cancer antigens by DCs, IFNγ mediated activation of CTL, M_1 Mφ and granulocytes, and expression of co-stimulatory molecules on T cells, cancer cells are eliminated by CTL and NK (natural killer) cells[4,60]. Under the constant immune pressure (continued deletion of cancer cells recognized by the immune system), some tumor cells undergo genetic and epigenetic changes (immune editing), enabling them to avoid immune attack, and sometimes immune cells are co-opt to favor immunosuppression.

During the equilibrium phase, the adaptive immune system holds the tumor in a state of functional dormancy; the environment is balanced between anti-tumor and tumor-promoting signals[60]. The molecular mechanisms that trigger immune-mediated tumor dormancy are so-far poorly understood, but some studies show that tumor antigen-specific T cells can arrest the growth of tumors by secretion of antitumor cytokines[60]. To detect occult cancer in equilibrium, is a challenge in the clinic.

Tumor escape occurs as neoplastic cells evade immune surveillance and the TME provides a survival advantage for neoplastic cells. Such "tumor adaptation" includes the selection of tumor antigens with low immunogenicity (not recognized by immune cells), loss of tumor cell MHC molecules and increased tumor cell survival (reduced receptors for apoptotic signals), and upregulation of immunosuppressive mechanisms such as immune checkpoints (Chapter 1.3.2)[60]. Tumor and stromal cells secrete cytokines (e.g. IL-10 and TGF-β) that induce a immunosuppressive, T_H2-polarized immune response hampering the cytotoxic and proliferative capacity of T cells, and increases their expression of co-inhibitory receptors[69].

Myeloid immune cells within the TME have great plasticity and can be modulated towards a M_2 immunosuppressive phenotype, antigen-presentation by DCs suppressed, and MDSCs recruited[69]. Foxp3+ CD4+T cells (T_{reg}) are hi-jacked by tumors, increase in numbers at the tumor site, and play an important role in suppressing CTL activity. Enhanced angiogenesis enables tumor progression and matrix remodeling may contribute to a dense stroma which inhibits the anti-tumor response[60,78].

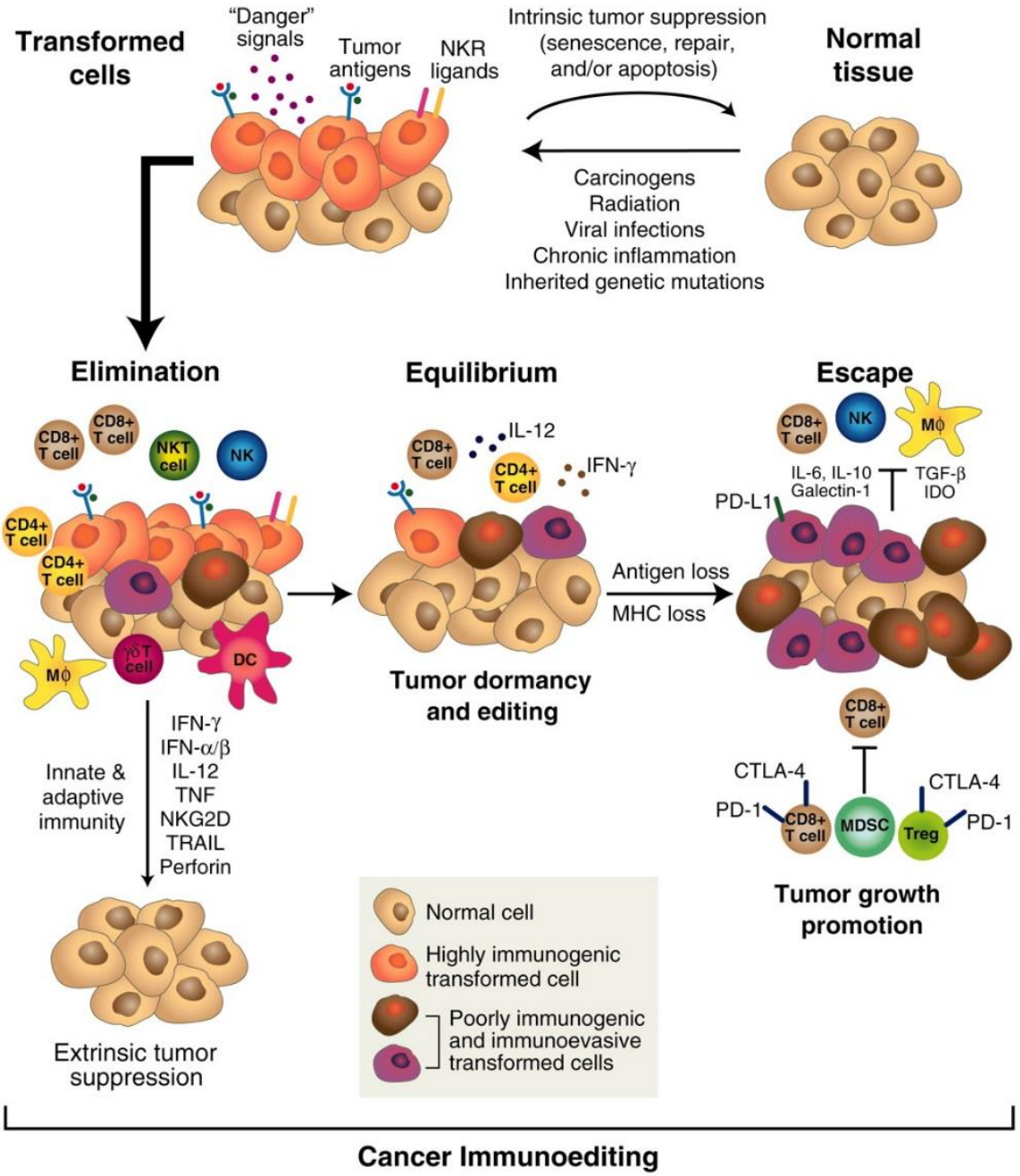


Figure 3 The three E's of immunoediting. (See 1.2.5) As published in[5]. Permission obtained from AAAS©.

1.2.6 Quantifying the immune contexture

The composition of immune cells in solid tumors can vary according to type and stage of cancer, and between patients with the same cancer type. Furthermore, the type, density, location and functional orientation, termed "the immune contexture", has in recent years been demonstrated to influence cancer patient outcome[79–82]. Studies have shown that immune cells in general have distinctive localizations in cancer[62,83]. Myeloid cells are in general found both in the invasive margin (IM) and central parts of tumors (CT), while T cells are mainly located in the IM, but can also infiltrate the CT[62,83]. NK-cells are mostly found in the stroma, while B-cells reside in the IM/TLS[62,83]. One large meta-analysis of 20 different cancer types showed that high infiltration of CD8+ T lymphocytes and a cytotoxic, CD8+/T_H1 signature in primary tumors correlate with good prognosis in most tumors[62,82,84]. However, in other cancers, these factors are correlated with poor prognosis[62]. The density of TLSs and mature DCs have been found to correlate with an effector memory/T_H1 phenotype and favorable clinical outcome in several cancers, but no universal method for evaluating TLSs exists[85]. A high ratio of T_{reg} to effector T cells is generally associated with poor outcome, while for other T_H cell subsets and myeloid cell populations, the clinical impact is less consistent and depends on tumor type and stage[62].

Contrary to most previous approaches for prediction of cancer patient outcome, the Immunoscore is an approach to quantifying immune factors in the TME, which can be of prognostic value in cancer patients, independent of established prognostic factors such as pathological stage[78]. The AJCC/UICC-TNM classification describes the degree of tumor progression at the time of the surgical resection, and is used to estimate patient outcome. It is currently the most important factor for predicting postoperative cancer patient prognosis and is the major rationale for individual treatment decisions, e.g. adjuvant treatment. However, TNM-classification relies on the assumption that disease progression and prognosis is tumor cell-autonomous, and clinical outcome may vary significantly within each pathological stage[86]. Increasing evidence suggests that quantifying the immune contexture may provide valuable prognostic information, supplementing the TNM staging and taking into account the balance between the invasive tumor process and the host defense system[87].

Initially developed for colorectal cancer (CRC) patients, Galon et al. designed the "Immunoscore", in which the *in situ* density of CD3+ (pan-lymphocyte marker), CD8+ cytotoxic and

CD45RO+ memory T cells and granzyme B was assessed by IHC both at the tumor center (CT) and invasive margin (IM)[79]. In both stage I-II (n=602) and stage I-IV (n=599) patients, the Immunoscore was the only predictor of patient survival superior to the TNM-classification staging method[81,88,89]. The Immunoscore (Figure 4) provided a scoring system ranging from I0 (low density of both cell types in both regions) to I4 (high density of both cell types in both regions)[80]. Highly significant and dramatic differences in disease-free survival (DFS), overall survival (OS) and disease-specific survival (DSS) were observed between I0 and I4 patients[80]. Furthermore, improved accuracy of prediction was obtained with the combined analysis of CT plus IM versus single-region analysis[80].

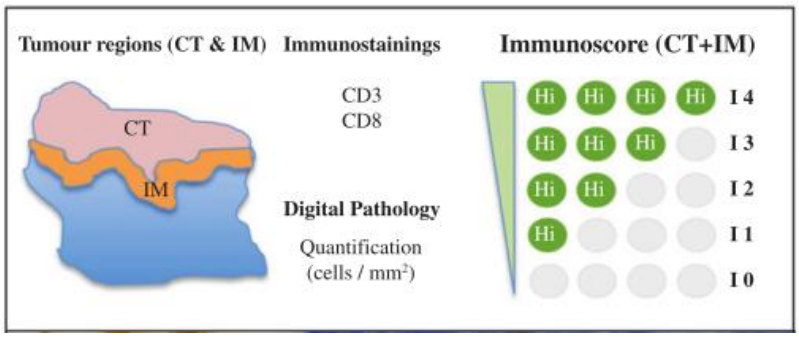


Figure 4 Immunoscore. Quantification of CD3+ and CD8+ TILs at the tumor center (CT) and invasive margin (IM)[87].

The method is simple and considered applicable in the clinical setting, and an international consortium was initiated to validate and promote the Immunoscore in routine clinical settings[87,90]. Results from an international multi-center study, evaluating the Immunoscore (IM) methodology in 1336 colon cancer patients, was presented at ASCO 2016, confirming an independent prognostic impact on time-to-recurrence[91]. Another large study found a semi-quantitative evaluation of TILs by simple H&E staining to be strongly associated with improved prognosis in CRC, however, there may be benefits of assessing TIL (tumor infiltrating lymphocyte subsets[86,92].

Evidence indicate that TIL infiltration in breast cancer tissue, evaluated by H&E sections, provide prognostic and potentially predictive values, particularly in triple-negative and human epidermal growth factor receptor-2-overexpressing breast cancer[93,94]. An international working group has published a guideline for evaluation of TIL on H&E-slides, and the approach has been further developed and validated[93,94].

Hence, the increased understanding of the complex interaction between tumors and the immune response has sparked a search for simple and pragmatic methods of quantifying the immune contexture in cancers, in order to more precisely predict patient outcomes, and potentially also therapy response[95].

1.3 Immune prognostic biomarkers in NSCLC

As in other cancers, the immune microenvironment can play dual roles in NSCLC progression. Two thirds of immune cells in the NSCLC TME are lymphocytes (80% T cells), while tumor-associated macrophages represent around 1/3, and NK cells and DCs are scarce[96]. Most immune cells are present in the stroma, and few within tumor islands[96]. An increasing amount of evidence has demonstrated that the NSCLC immune contexture can have a prognostic impact, as previously reviewed [97,98]. CD8+ TILs have most consistently been correlated with improved survival in NSCLC, along with CD3+, and in some studies CD4+ T cells[97–100]. An elevated Foxp3+ T_{regs} level and T_{reg} to CD3+ ratio has generally been associated with poor survival[97,101]. The prognostic impact of B cells, T_{H17}+ and NK cells is not clear, and reports on Mφs and neutrophils show diverging results[97,102–104]. The presence of immune cells organized in intratumoral TLSs, as indicated by mature DCs have been shown to correlate with a high infiltration of tumor infiltrating lymphocytes (TILs, primarily of the effector-memory type), to genes related to T cell activation, T_H1 phenotype and cytotoxic orientation, and a favorable prognosis[105].

Employing a similar strategy as in CRC, our research group has recently demonstrated a strong and independent prognostic impact of the density of stromal CD8+ TILs, supplementing the TNM-classification in order to predict postoperative prognosis for resected stage I-IIIA NSCLC patients[106].

1.4 Description of immune prognostic biomarkers explored in this thesis

1.4.1 CD45RO+ memory T cells (Paper I)

Memory T cells are the fraction of activated T cells which are long-lived and can readily elicit an effective protective immune response upon re-exposure of a pathogen, so-called "immunologic memory"[107]. Memory T cells are classically distinguished by the expression of the CD45RO isoform, and represent a heterogeneous population of cell subsets, which home to different sites in the body and have varying phenotypes and cytokine patterns (Chapter 1.2.4)[108].

It has been hypothesized that memory T cells have a long-lasting anti-tumor capacity, critical for the induction of killing or suppressing tumor cells. Further, a prognostic impact of tumor-infiltrating CD45RO+ T memory cells has been demonstrated in several cancer types[80,88,109–117]. An important role of memory T cells has been suggested with respect to improved NSCLC outcome. However, studies examining the prognostic impact of the *in situ* expression of memory T cells, applying the routinely used and validated antibody CD45RO, are lacking[105,118–120].

1.4.2 Immune checkpoints: CTLA-4 and PD-1/PD-L1 (Paper II and III)

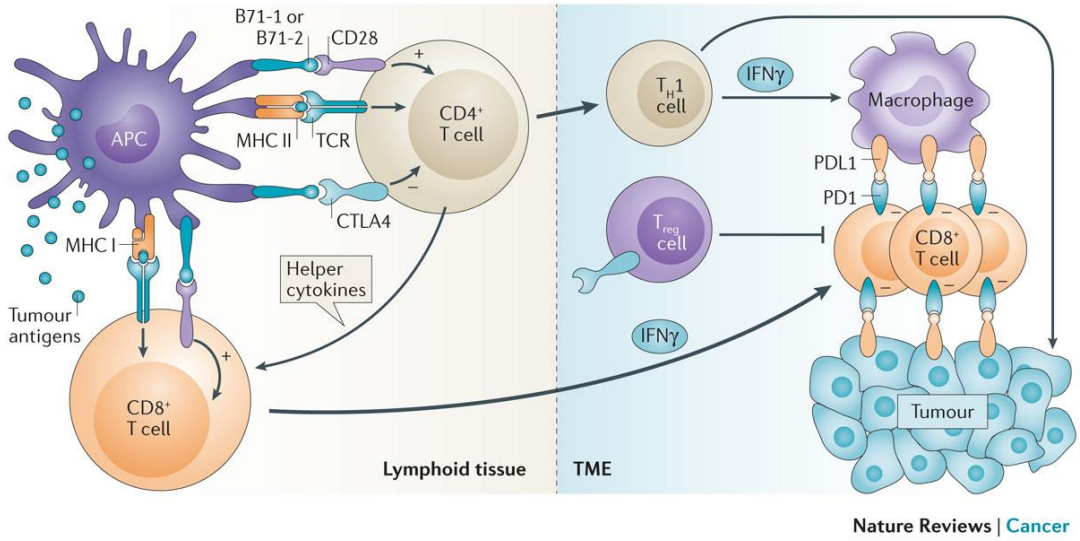
The inhibitory signals which are crucial for physiological fine-tuned regulation of T cell activation and functions, referred to as immune checkpoints, are exploited by tumors to restrain anti-tumor immune responses. Overexpression of inhibitory ligands or receptors involved in immune checkpoint pathways in tumor cells or TME is an important tumor immune resistance mechanism[70]. The two most studied immune checkpoints in cancer are CTLA-4 and the PD-1/PD-L1 pathway.

CTLA-4 is a transmembrane protein rapidly expressed on T cells upon activation (predominantly CD4+), elicited by the engagement of TCR to pMHC and ligation of co-stimulatory CD28 on T cells with B7 ligands (CD80 or CD86) on APCs[39]. CTLA-4 is constitutively expressed on T_{regs}. The binding of CTLA-4 to CD80/CD86 functions as a negative feedback signal which inhibits early stages of T cell activation, preventing immune hyperactivation, but also reinforces the immunosuppressive activity of T_{regs}[39] (Figure 5).

The transmembrane protein PD-1 is also expressed on T cells upon activation, although in a more delayed fashion, and is thought to directly inhibit T cell effector functions, predominantly in the

effector phase[121]. The PD-1 ligand (PD-L1) is commonly overexpessed by tumor cells and infiltrating leukocytes, induced by inflammatory mediators such as IFNγ produced by cells in the TME (Figure 5)[39]. PD-L1 may also mediate inhibition of activated T cells through CD80 and suppressive "backwards signals" in APCs[121]. Elevated expression of PD-1 (and other co-inhibitory molecules) due to chronic stimulation by tumor antigens is also thought to be a marker of a T cell dysfunctional state, similar to T cell exhaustion in chronic infection[122]. Even though the CTLA-4 is thought to exert its T cell suppressive function mainly in SLOs, and PD-1 in peripheral tissues, recent evidence has indicated that CTLA-4-blockade in cancer may inactivate tumor-infiltrating T_{reg} cells outside of lymph nodes[39]. Hence, both checkpoints may play important roles in the TME.

Figure 5: Immune checkpoint pathways in cancer. The PD-1/PD-L1 checkpoint is thought to mainly affect CD8+ T cells, while CTLA-4 predominantly regulate CD4+ T_{H1} and T_{reg} cells. Both CD4+ and CD8+ T cells are activated by the recognition of tumor antigen presentation MHC-molecule (signal 1) and co-stimulatory pathways (signal 2) in the context of pro-inflammatory cytokines. Tumor-specific activated T cells differentiate, proliferate and migrate to sites where tumor antigen is present and cytolytic activity commences. Within hours to days, activated T cells express the co-inhibitory receptor PD-1. Immune cells induce an inflammatory TME, e.g. by secretion of IFNγ, which stimulates antitumor cytolytic activity and tumor antigen display, but also induces PD-L1 secretion by tumor and stromal cells in the TME. Engagement of PD-1+ T cells with PD-L1 inhibits T cell function. As published in[123]. Permission obtained from Nature Publishing Group©



2 AIM OF THESIS

The general aim of the work included in this thesis was to investigate the relevance of important immune biomarkers, expressed by malignant and non-malignant cells, in the tumor microenvironment of NSCLC tumors with regard to patient outcome, thereby assessing aspects of the natural process of NSCLC disease progression and metastasis.

More specifically, the aims of this thesis are:

- By immunohistochemistry, investigate the *in situ* prevalence and patterns of expression of acknowledged important immune markers in tumor cells and surrounding stroma.
- Examine the expression and prognostic impact of these markers in primary tumors and lymph node metastases, in order to explore similarities and differences of clinical importance.
- Asses the prognostic impact of these markers alone and in relation to other prognostic factors in NSCLC, including pathological stage.
- Evaluate the markers as candidates for novel methods of quantifying the NSCLC immune infiltrate, in order to supplement the current prognostic tools available for clinicians and thus potentially to improve clinical decision-making and patient survival.

3 MATERIALS AND METHODS

3.1 Patient cohort

All consecutive patients who underwent radical resection for clinical stage I-IIIA NSCLC at the University Hospital of North Norway (UNN), Tromsø, and Nordland Hospital (NH), Bodø from 1990 through 2010 were identified retrospectively, through searches in the archives of their respective Departments of Pathology (n=633). Available primary tumor blocks were collected and patient demographic and clinicopathological data were compiled into a database and de-identified. A key is accessible within our research group, with the intent of updating survival data.

The database was first established in 2005 and included 335 patients surgically treated between 1990 and 2005[124]. The papers in this thesis include analysis of survival data from the third and most recent update (follow-up data as of October 1 2013), in which 219 patients treated between 2005 and 2011 were added. Thus, 536 patients with complete medical records and adequate paraffin-embedded tissue blocks were eligible (Figure 6). In addition, of the 172 patients with N+ disease included in the database, we collected tissue specimens from the 143 patients whose tumor specimens were available and adequately paraffin-embedded. Demographic, clinical and histopathological variables were retrospectively collected and are presented in Table 2.

The tumors collected before 2010 were initially staged according to the 6th edition International Union Against Cancer (UICC) TNM classification, but were reviewed and restaged according to the 7th edition[26] upon its implementation in 2010. Tumors were histologically classified according to the 2004 WHO guidelines on classification of lung cancer[125]. We have not histologically reassessed tumors according to the 2011 IASC/ATS/ERS lung adenocarcinoma and 2015 WHO lung cancer recommandations[2,18]. Hence, we have continued to use the entity LCC in our studies, but were able to reclassify BACs (bronchioloalveolar carcinoma) of ≤ 3cm as AIS and excluding these from the analyses, without any new assessment of tumors. A histological review and re-classification of all tumors in the database is ongoing.

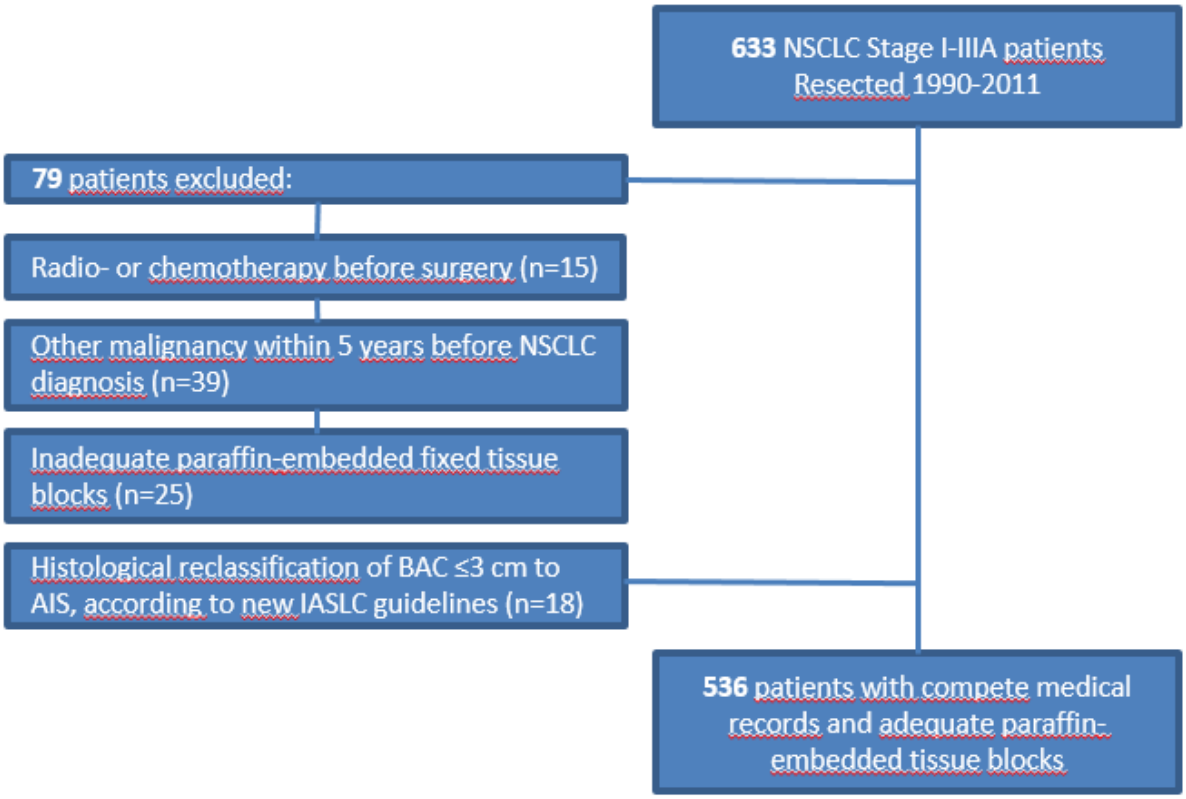
Table 2 Clinicopathological variables as predictors of disease-specific survival (DSS) in 536 NSCLC patients (univariate analyses; log-rank test, unadjusted Cox proportional hazard ratios).

	All patients					Squamous cell carcinoma					Adenocarcinoma				
	N(%)	5 year	Median	HR(95% CI)	P	N(%)	5 year	Median	HR(95% CI)	P	N(%)	5 year	Median	HR(95% CI)	P
Age					0.711					0.654					0.505
≤65	227(42)	57	127	1		106(37)	64	235	1		102(51)	48	54	1	
>65	309(58)	58	NA	0.95 (0.73-1.24)		183(63)	66	NA	0.91(0.61-1.36)		99(49)	49	57	0.87(0.59-1.3)	
Sex					**0.026**					0.108					**0.050**
Female	170(32)	63	190	1		73(25)	73	NA	1		83(41)	56	190	1	
Male	366(68)	55	88	1.4(1.06-1.84)		216(75)	63	235	1.49(0.96-2.31)		118(59)	43	51	1.5(1.01-2.23)	
ECOG perf. status					**0.015**					0.158					**0.003**
0	310(58)	62	235	1		158(55)	69	235	1		122(61)	56	NA	1	
1	190(35)	52	71	1.45(1.09-1.93)		110(38)	61	114	1.47(0.97-2.23)		67(33)	40	50	1.57(1.02-2.4)	
2	36(7)	48	36	1.61(0.83-3.09)		21(7)	67	NA	1.08(0.45-2.6)		12(6)	17	25	3.25(0.96-11.03)	
Smoking					**0.039**					0.19					0.68
Never	17(3)	44	20	1		7(2)	50	19	1		9(5)	44	21	1	
Previous	342(64)	62	235	0.56(0.25-1.24)		182(63)	69	235	0.58(0.14-2.37)		125(62)	50	68	0.69(0.26-1.84)	
Present	177(33)	51	71	0.75(0.33-1.7)		100(35)	60	114	0.82(0.2-3.41)		67(33)	45	57	0.73(0.27-1.99)	
Weightloss					0.961					0.689					0.536
<10%	480(90)	58	127	1		257(89)	66	235	1		184(92)	49	57	1	
≥10%	55(10)	59	NA	0.99(0.63-1.56)		32(11)	62	NA	1.14(0.57-2.28)		17(8)	40	47	1.24(0.59-2.63)	
Surgical procedure					**<0.001**					**<0.001**					**<0.001**
Wedge/Lobectomy	394(74)	63	190	1		197(68)	72	235	1		161(80)	54	104	1	
Pulmonectomy	142(26)	42	30	1.98(1.43-2.74)		92(32)	50	35	1.99(1.28-3.09)		40(20)	25	24	2.66(1.46-4.84)	
Margins					0.129					0.252					**0.018**
Free	489(91)	59	190	1		257(89)	67	235	1		189(94)	50	68	1	
Not free	47(9)	47	57	1.39(0.85-2.29)		32(11)	57	114	1.39(0.73-2.63)		12(6)	0	35	2.33(0.81-6.69)	
Tstage					**<0.001**					**<0.001**					**<0.001**
1	168(31)	72	235	1		83(29)	78	235	1		74(37)	67	190	1	
2	265(49)	57	91	1.74(1.3-2.32)		147(51)	66	NA	1.88(1.22-2.89)		94(47)	43	47	1.94(1.27-2.95)	
3	97(18)	36	30	2.84(1.87-4.31)		56(19)	46	33	2.93(1.62-5.31)		31(15)	16	25	3.48(1.76-6.9)	
4	6(0)	20	15	4.89(0.89-26.9)		3(1)	0	10	17.41(0.22-1371.77)		2(1)	50	13	1.76(0.23-13.27)	
Nstage					**<0.001**					**<0.001**					**<0.001**
0	364(68)	69	235	1		198(69)	77	235	1		133(66)	60	190	1	
1	118(22)	36	35	2.76(1.93-3.94)		73(25)	45	35	3.26(1.99-5.35)		39(19)	25	30	2.41(1.38-4.2)	
2	54(10)	21	19	4.23(2.43-7.37)		18(6)	18	13	7.12(2.44-20.77)		29(15)	23	24	2.88(1.42-5.82)	
Pathological stage					**<0.001**					**<0.001**					**<0.001**
I	256(48)	72	235	1		127(44)	82	235	1		105(52)	65	190	1	
II	194(36)	53	84	1.89(1.42-2.51)		126(44)	60	114	2.5(1.66-3.77)		56(28)	34	43	2.07(1.3-3.28)	
IIIA	86(16)	20	17	4.58(2.87-7.32)		36(12)	23	15	7.15(3.23-15.84)		40(20)	16	24	3.37(1.8-6.33)	
Histology					**0.040**										
SCC	289(54)	65	235	1											
ADC	201(37)	48	57	1.43(1.08-1.89)											
LCC	46(9)	50	83	1.29(0.8-2.08)											
Differentiation					**<0.001**					**0.033**					**0.006**
Poor	231(43)	49	51	1		104(36)	57	84	1		81(40)	38	43	1	
Moderate	240(45)	63	190	0.67(0.5-0.89)		155(54)	70	235	0.63(0.41-0.97)		85(42)	50	68	0.69(0.44-1.07)	
Well	65(12)	70	NA	0.44(0.29-0.66)		30(10)	72	NA	0.47(0.24-0.94)		35(18)	69	NA	0.36(0.21-0.63)	
Vascular infiltration					**<0.001**					**0.029**					**0.012**
No	437(82)	62	235	1		231(80)	69	235	1		172(86)	52	71	1	
Yes	97(18)	38	35	1.89(1.29-2.78)		58(20)	53	71	1.65(0.97-2.82)		27(13)	26	27	1.9(1-3.62)	
Missing	2(0)										2(1)				

Abbreviations: ADC, adenocarcinoma. ECOG perf.status, Eastern Cooperative Oncology Group performance status. HR, hazard ratio. LCC, large cell carcinoma. N, number. stage, Nodal stage. SCC, squamous cell carcinoma. Tstage, Tumor stage.

As illustrated in Figure 6, exclusion criteria were 1) radio- or chemotherapy prior to surgery (n=15), 2) other malignancy within 5 years before NSCLC diagnosis (n=39), 3) inadequate paraffin-embedded fixed tissue blocks (n=25). In addition, histological classification of BAC ≤3 cm were reclassified to AIS, according to new IASLC guidelines[2] (n=18).

Figure 6 Inclusion and exclusion criteria.



3.1.1 Discussion, Patient cohort

Exclusion criteria 1) and 2) were adapted to avoid bias as these mechanisms might introduce changes in the tumor microenvironment not caused by the lung cancer tumor. Radio- or chemotherapy may introduce necrosis of malignant cells and thereby recruit immune cells, and other (previous or synchronous) malignancy might have introduced changes in the hosts response to tumor.

Collection of quality pathological and clinical data is, of course, essential. When clinical data is collected retrospectively, one must take into consideration the potential information bias introduced, especially with regard to clinical follow-up data taken from patient files. Cause of death can be biased by subjective interpretation, but was critically reviewed when collecting data from patient files.

Hence, we have primarily used tumor-specific death as censor for survival analyses in our analyses of prognostic impact, while others find overall survival more valuable[126].

If donor samples have been collected over a long period of time, diagnostic criteria may have changed. In addition, interobserver differences in tumor classification may exist, and introduce variability in how patients are histologically classified. In our database, an expert pathologist reviewed all sections before including tissue core in the TMA, and when diagnostic classification criteria changed, as recommended[126].

Changes in post-surgical treatment over time and between individuals represent an important confounder. Norway has a universal public health service financed by taxation, equally accessible to all residents. Norwegian national guidelines are easily available, and relatively uniform treatment can be expected. While our dataset does not include information on clinical staging (e.g. PET) or palliative treatment, data on adjuvant radiotherapy (entire cohort) and adjuvant chemotherapy (patients included 2005-2010) was collected and analyzed.

To explore the significance of time and treatments, we assessed PD-Immunoscore according to adjuvant chemo- and radiotherapy and in two time periods in Paper II. Interestingly, the PD-Immunoscore was a significant prognosticator (or trend, most likely due to the small number of patients in this subgroup) in all subgroups (Table 3). Additionally, adding either variable as covariates in multivariate analyses did not change the outcome.

Table 3 PD-Immunoscore as predictor of DSS in subgroups.

Adjuvant chemotherapy		**Adjuvant radiotherapy**		**Time period**	
Yes	No	Yes	No	1990-2004	2005-2010
43	167	67	429	326	210
0.005	0.020	0.058 (trend)	<0.002	0.004	0.001

3.2 Tissue microarray

The construction of tissue microarrays (TMA) was initially described in 1998, and involves transferring small representative tissue cylinders, most commonly 0.6 mm diameter, from a "donor" tissue block into empty "recipient" paraffin blocks in a systematic pattern[127]. Most TMAs are made from archived formalin-fixed paraffin-embedded (FFPE) resections or larger biopsies, but fine-needle biopsies, cytologic cell block material, cell lines, and frozen tissue can also be used[128–131]. Hundreds of cores can be arrayed onto each TMA recipient block, and each "recipient" TMA block may subsequently be sectioned and produce up to 300 slides[132].

TMAs are being used for multiple purposes, often in oncology research: in basic/translational research, for analysis of biomarker frequency or protein expression and subcellular localization (prevalence TMAs); analyses defining the relationship between biomarker expression and clinicopathological features, including different stages of disease (progression TMAs) and disease prognosis (prognostic TMAs). Evaluation of biomarkers in TMAs collected from homogenous patient populations included in clinical trials can also be associated with treatment outcome data (predictive TMAs)[133]. In addition, TMAs containing multiple tumors and normal tissues are used for validation of the sensitivity and specificity of antibodies, and quality assurance in IHC[134].

3.2.1 TMA construction

All blocks were sliced into whole tissue sections and stained with hematoxylin and eosin (H&E). An experienced pathologist histologically reviewed slides for tumor content, quality and histological subtype, and the most representative areas containing viable neoplastic epithelial cells and tumor stroma were carefully selected, marked on the H&E slide and sampled for tissue microarray blocks. Two core samples from different areas of neoplastic tissue and two of tumor stroma from each patient's primary tumor were included in the TMAs. For patients with locoregional metastatic lymph nodes (N+ disease), two core samples from one metastatic lymph node were included. In case where more than one lymph node metastasis was available, the one deemed most suitable for TMA by the pathologist was chosen.

The TMAs were assembled using a manual tissue-arraying instrument (MTA-1, Beecher Instruments, Silver Springs, MD, USA). The methodology is well documented and has been previously

reported in detail[135]. Briefly, we used a thin-walled biopsy needle and stylet to create holes in a recipient paraffin block, and to sample and transfer 0.6 mm diameter cylindrical tissue cores from the donor block to the recipient block at defined array coordinates (Figure 7). Normal tissue localized distant from the primary tumor was included in the TMA to allow negative/positive tissue staining control, and a non-symmetric array was chosen to enable orientation. Multiple 4-µm sections were cut with a Micron microtome (HM355S) and one section was stained by H&E to assess the tumor content in each core. Missing cores could thus be replaced in subsequent TMA blocks, to ensure that four cores from each patient were available for analyses. Twelve primary tissue and three metastatic lymph node TMA blocks were constructed, including up to 288 tumor cores each.

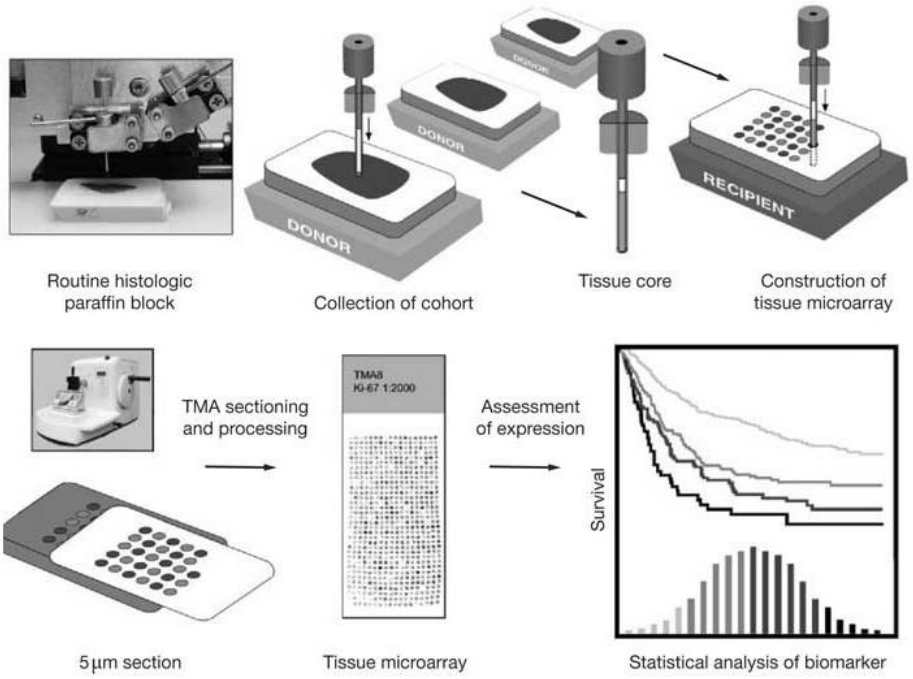


Figure 7 Construction of a tissue microarray. Paraffin-embedded, formalin-fixed tissues are collected. Cores (0.6 mm) from representative areas of tumor and stroma are punched from donor blocks and transferred into a recipient block in an array structure. Sections of the TMA are cut and transferred to slides for immunohistochemistry processing. Biomarker expression assessment (scores) are linked to clinicopathological information. As published in[136]. Permission obtained from Nature Publishing Group©.

3.2.2 Tissue microarray: advantages

The most commonly applied TMA technique is immunohistochemistry (IHC), but most histological and molecular techniques available for whole tissue sections (WTS) can be applied to TMA sections, including *in situ* hybridization techniques and immunofluorescence methods[133]. As many as 300 biomarker analyses may be produced from consecutive slides on one TMA array and may be analyzed simultaneously, saving valuable tissue, time and reagents[132]. Thus, a large number of tissue specimens may be rapidly analyzed, using tissues routinely processed in surgical pathology. Moreover, the regular shape and small diameter of each specimen, and highly organized array pattern permits reliable allocation of clinical data to the individual tissue spots[137].

Another important advantage of the TMA technique is its maximal standardization; technical sources of variation are almost eliminated as all cases and control tissues, are stained under identical experimental conditions[138]. Subjective interpretation is improved compared to WTS, as observers may directly compare staining intensities between multiple tumors on each TMA slide, and areas to be scored are small and predefined[139]. In addition, different biomarkers can be evaluated in consecutive sections in order to explore the regional distribution of multiple biomarkers in the same tumors[139].

To sum up, by using the TMA technique, the parallel *in situ* detection of DNA, RNA and protein targets in pathologically well-defined tissues can be linked with long-term clinical follow-up information, making TMA a powerful high throughput method for performing biomarker analysis of large patient cohorts[138].

3.2.3 Tissue microarray: challenges

Many of the challenges faced by the TMA technique are essentially the same as for large tissue sections; preanalytic factors such as ischemic time, fixation type and fixation time show variability, and analytical factors such as intra- and interobserver differences during scoring also significantly affect the performance characteristics of the TMA analyses[126]. Some major challenges when utilizing the TMA technique are described below. Issues concerning experimental conditions are described in chapter 3.3.

3.2.3.1 Pre-analytical phase

The degradation of DNA, RNA and proteins starts as soon as blood supply is interrupted, but limiting the time from ischemia to fixation is difficult to standardize[140]. Fixation is performed in order to maintain cell morphology, tissue architecture and antigenicity of target epitopes. Delayed fixation may cause increased, decreased or de-localized immunoreactivity during IHC staining[141]. Fixation with formalin causes proteins to cross-link and prevents microbial degradation and autolysis, but formalin penetration depends on the size, volume and composition of the tissue, inevitably causing variations in fixation between tissue cores in the TMA[126].

3.2.3.2 Analytical phase

During the process of microscopically analyzing the stained TMA, the skills of the scorer are essential. The observer must, quickly and correctly, assess cell types, discern non-malignant from malignant cells and tissue compartments (tumor versus stroma). An impressive concordance between an expert pathologist and individuals with only rudimentary training has been reported, but different levels of analytical difficulty will exist for different antibodies and targets[126].

3.2.3.3 Representativity

Ever since the first report in 1998, a major objection against TMA has been the issue of tumor heterogeneity; whether the small cores are representative for donor tissue or not, and what size and number of cores are optimal[127]. First, it is important to select the most representative areas of each tumor for sampling[142]. Next, it has been suggested that using larger tissue cores, or multiple cores, from the same donor tissue might enhance representativity. Many studies have applied TMA technique to reproduce previously well-established associations between molecular alterations and clinical outcome, validating the reliability of the TMA method[139,142,143]. Also, several studies have evaluated the representativeness of TMAs compared to corresponding WTS applying IHC technique. Along with its superior staining and analytical standardization, most have found that, as long as sufficiently large numbers of tissue samples are included in the TMA, between two and four 0.6 mm diameter cores is sufficient to identify associations between molecular alterations and outcome[138,144].

Few studies have assessed heterogeneity for different biomarkers in NSCLC tissue. Some have performed TMA validation by WTS, supporting the validity of TMA analysis in NSCLC studies with replicate 0.6-1.35 mm diameter cores with acceptable concordances[145–148]. Of note, biomarkers expressed in tumor cells only, were assessed. Small studies in other cancers have found TMA technology appropriate for analyzing both inflammation and tumor cell markers, but moderate concordance with WTS was reported, and the importance of selecting appropriate areas of scoring for inflammatory markers was highlighted[149,150].

An interesting aspect in this debate, is the assumption that WTS is a "gold standard" with regard to representativity and tumor heterogeneity. In fact, if considering a 3 cm diameter tumor ($14cm^3$), one WTS (3cm x 2cm x 4µm=$0.0024cm^3$) represents about 1/6000 of the tumor, while a 0.6 mm tissue core ($0.0000011\ cm^3$) represents about 1/2000 of the WTS. Hence, the correlation between TMA cores and WTS is larger than that between WTS and the tumor. That being said, concordance of biomarkers between TMA cores and WTS is marker-specific and dependent on tissue type[151]. Larger size and number of cores may be appropriate for the analysis of antigens with limited tissue expression, excessively heterogeneous distribution or location-dependent expression[144]. Finally, it is important to be aware that TMA technology is not intended for making individual case decisions, but is a population-level research tool[132].

Table 4 Advantages and disadvantages of the tissue microarray technique.

Advantages	Disadvantages
Permits rapid staining and analysis of large number of cases	TMA construction is time consuming and demands experience
Array patterns permits reliable allocation of clinical data to individual cores	10-15% of cores are expected to be missing
Standardized experimental conditions	Heterogeneous tissue may be underrepresented
Saves time, tissue and reagents	Tissue content may change over the length of the core.
Archived FFPE tissue can be used	Not suitable for individual diagnosis
Inclusion of normal tissue as internal control	
Can be easily shared with other institutions	

3.2.4 TMA: Discussion

Performing analyses of WTS in our studies would be advantageous, as it is what is currently available for diagnostic purposes in the clinic, and it would permit evaluation of intratumor heterogeneity and number of fields-of-view or TMA cores required to find associations between biomarkers and outcome. However, in the studies included in this thesis, we have decided against staining and analyzing WTS supplementary to TMAs, as we argue that our TMA material is adequately representative with regard to size and number of cores. When a biomarker is heterogeneously expressed, the estimated effect size and ability of a study to detect a specific effect size (power) are attenuated, increasing the probability of a type 2 error[152]. By including a large number of patients and two to four cores per tumor we have to some degree decreased the influence of intraindividual variation, producing more reliable estimates. Also, the scoring agreement between cores was analyzed and found relatively high (Paper II, ICC 0.726-0.917), especially for primary tumors, in which our main results are found. Finally, our study must be seen as hypothesis generating, demanding further validation. Optimal number and size of cores should optimally be determined by each individual biomarker and by choice of positive threshold level, before potential implementation into the clinic.

3.3 Immunohistochemistry

Immunohistochemistry (IHC) refers to the detection of antigen within tissue sections by means of specific antibodies binding to the antigens. The method is considered the "gold standard" for evaluation of *in situ* protein expression in tissue sections. It is a widely used supplement in diagnostic pathology to obtain an accurate diagnosis and to analyze prognostic and predictive biomarkers, and is an important method in basic research and drug development[153,154].

The indirect IHC method involves the following steps; a) the application of a specific primary antibody which binds to the antigen of interest, b) a secondary, enzyme-conjugated antibody, specific against the primary antibody, is incubated with the tissue and binds to the primary antibody, c) a chromogen (e.g. DAB) is applied to visualize the antibody-antigen complex[155] (Figure 8).

3.3.1 IHC procedure

All sections, including multi-tissue TMAs, were deparaffinized with xylene and rehydrated with ethanol. On-board antigen retrieval by incubation with Ventana cell conditioning reagent for 24-64 minutes (not CD45RO). Endogenous enzymes were quenched prior to adding the primary antibody, when recommended by the antibody manufacturer (CD8, PD-1, CTLA-4 only). Automated staining using the Discovery ULTRA or Benchmark ULTRA staining platform was used for all IHC procedures. The antibodies and IHC procedure applied in the studies in this thesis are presented in Table 5. Upon developing slides and applying chromogen, counterstaining with hematoxylin and a bluing reagent to visualize nuclei and overall tissue architecture was performed. Finally, slides were dehydrated, mounted and cover-slipped as in routing processing.

3.3.2 IHC advantages

The IHC method has many advantages. It allows the *in situ* assessment of the distribution and localization of specific cellular components in different compartments of tissue sections, and is relatively inexpensive. The method is established in most laboratories and can be performed on archived tissue. IHC slides can be processed and stained manually or in a high-throughput automated fashion.

3.3.3 IHC challenges

Variability in pre-analytic factors such as tissue collection, fixation (delay, time, type), tissue processing (paraffinization, drying, storage temperature, sectioning, mounting) and antigen retrieval method (pH, buffer, heat platform, time), non-specific site block and endogenous peroxidase block can affect tissue antigenicity. Also, the multi-step process of detecting antigens include variable analytical factors such as choice of antibody (clone, vendor, species, mono-or polyclonal), antibody concentration and incubation time/temperature, application of secondary antibodies and antigen detection methods[156]. Because standardization is crucial to achieve reproducible and reliable IHC test results, and thorough optimization of all steps of the IHC process are demanded experience on the part of technicians performing IHC experiments is demanded[156].

3.3.4 Antibodies

The antibody is the pivotal reagent in all IHC techniques, and antibody selection is a critical step in performing a reliable IHC study. Polyclonal antibodies are produced by immunizing animals, typically rabbits, with antigen, and the antibodies are generated by different B-cell clones. Hence, polyclonal antibodies bind to various epitopes on an antigen, and have slightly different specificities and affinities. Monoclonal antibodies, on the other hand, are generated by a single B-cell clone from one animal, and are therefore homogenously directed against a single epitope. Polyclonal antibodies can recognize multiple epitopes on the target molecule, and are therefore more robust reagents; variations in the pre-analytic processing of specimens influence the result less. As a consequence, they show a higher probability for detection in a range of different conditions, and false negative IHC results are infrequent. The drawback is that there is an increased chance for cross-reactivity with other proteins, producing false positive results. If not contaminated, monoclonal antibodies lack the variability of polyclonal antibodies, have high lot-to-lot consistency and are more specific, but are more likely to work in only one set of conditions, and false negative IHC results (weak signal) are more frequent[154,155].

Table 5. Antibodies and IHC procedures.

Antibody	Vendor	Catalog number	Clone	Host species and clonality	Primary antibody titer	Primary antibody time/temp	Secondary antibody	Positive control tissue	Negative control tissue
CD45RO	Ventana (Roche)	790-2930	UCHL-1	Mouse Monoclonal	Pre-diluted	20 min at 37°C	Ultramap anti-mouse HRP #760-4310, Ventana	Tonsil	Brain
CD8	Ventana (Roche)	790-4460	SP57	Rabbit Monoclonal	Pre-diluted	32 min at 36°C	Ultramap anti-rabbit HRP #760-4315, Ventana	Tonsil	Brain
PD-L1	Cell signaling Technology	13684	E1L3N	Rabbit Monoclonal	1/25 dilution	32 min at 37°C	Ultramap anti-rabbit HRP #760-4315, Ventana	Placenta	Brain, ventricle
PD-1	Abcam	ab52587	NAT105	Mouse Monoclonal	1/50 dilution	60 min at 37°C	Ultramap anti-mouse HRP #760-4310, Ventana	Tonsil	Brain, ventricle
CTLA-4 (CD152)	eBioscience	14-1529	14D3	Mouse Monoclonal	1/100 dilution	32 min at 37°C	OmniMap anti-mouse HRP #760-4310, Ventana	Placenta	Brain
PD-L1	Abcam	ab58810		Rabbit polyclonal	Could not be validated				
PD-L1	R&D systems	MAB1561	130021	Mouse Monoclonal	Could not be validated				
CTLA-4	Abcam	ab151773		Rabbit polyclonal	Could not be validated				

3.3.5 Antibodies: challenges

Antibodies should be specific, selective and reproducible in the context for which they are used, hence careful validation of antibody reagents is critical for correct results[154]. Accompanying the expansive increase in the availability of antibodies, and the use of IHC in research and diagnosis over the last 40 years, reliability, reproducibility and variations in protocols have become major challenges for IHC utilization[155]. Issues such as non-specific antibodies, strong background staining and weak target antigen staining must be overcome and optimization for dynamic expression ranges is important[157]. The antigen of interest must be identified in cells and structures with both low and high expression. A major disadvantage of IHC is that it is impossible to show that the staining corresponds with the protein of interest, hence, the interpretation of IHC results relies on the use of method controls and a general acceptance of what is considered to be appropriate staining according to medical literature[153]. The use of positive/negative control tissue is essential to evaluate antibody specificity, preferably including normal and pathologic tissue, preferably prepared as TMAs[158].

3.3.6 Antibody selection, controls and validation

For the studies presented in this thesis, we chose antibodies which had been successfully used by others, based on review of available literature and manufacturers' information and online databases. We used TMAs containing multiple different tumors and normal tissues as control tissue material to verify the specificity of the antibodies. The control TMA tissues were fixed and processed in the same manner as the study material. In addition, the study TMA included cores taken from tumor stroma, as well as normal lung tissue cores, and could be used for internal tissue control.

The antibodies used in Paper I (CD8, CD45RO) were subjected to validation by the manufacturer for IHC analysis on paraffin-embedded material, are both in routine clinical or clinical setting and are antibodies with high quality literature evidence[158]. These were validated on positive and negative tissues to gain appropriate signal-to-noise ratio (Table 5).

Little or diverging published NSCLC IHC data was available for the antibodies used in Papers II and III (PD-L1, PD-1 and CTLA-4). Therefore, in addition to in-house validation by the manufacturers and positive and negative tissue control, we performed further validation by transfectant plasmid cell lysates (in detailed described in Papers II and III) to select adequately performing antibodies. Once

successfully validating antibodies, IHC controls were run in accordance with general recommendations[158]. Firstly, control staining by omission of the primary antibody and replacing the primary antibody diluent was performed. Secondly, incubation with an isotype-matched control antibody, omitting the primary antibody, was performed.

3.3.7 Discussion: IHC

IHC procedures were performed by an experienced technician. Validation procedures were performed, adapted to the level required by existing recommendations[158]. Monoclonal antibodies were chosen for all studies presented in this thesis, which are expected to be more specific than polyclonal antibodies, but more likely to work in only one set of conditions, increasing the risk for false negative IHC results[154,159].

Challenges with regard to validity and reproducibility of novel immunological markers involved in T cell regulation, such as PD-1 and CTLA-4, are discussed in more detail in Chapter 5.

3.4 Microscopic evaluation of staining/scoring

Semi-quantitative IHC evaluation methods involves employing a relative, arbitrary scoring range, usually lacking a defined reference standard[155]. A major criticism against the manual semi-quantitative approach is that it is a subjective process which can be difficult to reproduce owing to inter- and intraobserver variability[160].

Applying semiquantitative scoring approaches including combinations of intensity and density have been introduced to overcome variations in scoring approaches between studies, such as the Allred-score, H-score and Immunoreactive score (IRS), but these methods also have their weaknesses (subjectivity, time consuming, loss of information/reduced sensitivity)[160–164]. Automated, digital imaging analysis is being used to an increasing degree, and has several advantages compared to manual/semi-quantitative scoring including improved reproducibility and speed of assessment, and creation of a continuous variable. However, manual exact count is still the "gold standard", against which other methods are compared[99].

3.4.1 Scoring procedures

In the studies presented in this thesis, samples were independently scored by two of the authors. We performed an initial evaluation of the distribution of staining in the cores and in the compartments to be assessed under the supervision of an experienced pathologist. Subsequently, a semi-quantitative score was established in a four-tiered ordinal scale (0-3), representing a density or intensity of staining. The scorers were blinded to the patient's clinicopathological variables, to outcome and to the scores of other markers.

Depending on the expected localization for each biomarker, staining was assessed in 1) the tumor epithelium, 2) the stromal compartment adjacent to the tumor epithelium, or 3) the intraepithelial immune cells, completely enclosed by tumor epithelial cells. Scoring details are presented in Table 6.

Staining predominantly found in cells morphologically consistent with tumor infiltrating immune cells (CD8, CD45RO, PD-1, stromal PD-L1) showed relatively homogenous intensities and were scored according to density (percentage of positive immune cells compared to the total amount of nucleated cells in the compartment). Staining of tumor epithelial PD-L1 and CTLA-4, and stromal

CTLA-4 was cytoplasmatic and/or membranous and relatively homogenous within each core, with variable intertumor staining intensity. These markers were scored according to intensity. The intraobserver reliability coefficients are reported in Table 3.

3.4.2 Discussion

The semi-quantitative scoring approach we have used is a relatively simple, pragmatic and low-cost approach. If supported by validation studies, it may be easily transferrable into clinical routine practice. Scores from two independent scorers were included to minimize subjectivity, and interobserver agreements varied from moderate to excellent for all biomarkers used (ICC, Table 6).

We established individual scoring approaches for each antibody to optimize for the scientific question at hand; analysis of prognostic impact. Unfortunately, inconsistency in study designs with regard to staining and scoring approaches make it difficult to draw conclusions when comparing results from different biomarker studies.

Table 6 Scoring.

	Primary tumor			Lymph node metastases	
	Tumor epithelial cells	Intraepithelial immune cells[a]	Stromal cells	Tumor epithelial cells	Intraepithelial immune cells[a]
CD8	NS	**T-CD8** Density (TILs)[b] 0=absent 1=1-5% 2=6-25% 3=26-50% Mean value 0.80 Maximum score Cutoff: High ≥2 ICC: 0.760	**S-CD8** Density (TILs)[b] 0=0.5% 1=6-25% 2=26-50% 3=>50% Mean value 1.55 Maximum score Cutoff: High ≥2 ICC: 0.878	NS	NA
CD45RO	NS	**T-CD45RO** Density (TILs)[b] 0=absent 1=1-5% 2=6-25% 3=26-50% Mean: 0.66 Cutoff: High ≥1 ICC: 0.642	**S-CD45RO** Density (TILs)[b] 0=absent 1=1-5% 2=6-25% 3=26-50% Mean: 1.24 Cutoff: High ≥2 ICC: 0.889	NS	NA
PD-L1	**T-PD-L1** Intensity 0=absent 1=weak 2=moderate 3=strong Mean: 1.12 Cutoff: High >1.25 ICC: 0.939	NA	**S-PD-L1** Density[b,c] 0=absent 1=1-49% 2=50-75% 3=>75% Mean: 1.45 Cutoff: High > 1.5 ICC: 0.879	**LN+ T-PD-L1** Intensity 0=absent 1=weak 2=moderate 3=strong Mean: 1.08 Cutoff: High >1.25 ICC: 0.951	NA
PD-1	NS	**T-PD-1** Density (TILs)[b] 0=absent 1=1-9% 2=10-50% 3=>50% Mean: 0.68 Cutoff: High >0.25 ICC: 0.891	**S-PD-1** Density (TILs)[b] 0=absent 1=1-24% 2=25-50% 3=>50% Mean 1.21 Cutoff: High >1 ICC: 0.900	NS	**LN+ T-PD-1** Density (TILs)[b] 0=absent 1=1-9% 2=10-50% 3=>50% Mean: 0.30 Cutoff: High >0.25 ICC: 0.826
CTLA-4	**T-CTLA-4** Intensity 0=absent 1=weak 2=moderate 3=strong Mean: 1.36 Cutoff: High >1.25 ICC: 0.917	NA	**S-CTLA-4** Intensity[c] 0=absent 1=weak 2=moderate 3=strong Mean: 2.08 Cutoff: High >2 ICC: 0.894	**LN+ T-CTLA-4** Intensity[c] 0=absent 1=weak 2=moderate 3=strong Mean: 1.21 Cutoff: High >1.25 ICC: 0.882	NA

[a]: Intraepithelial cells, defined as tumor infiltrating immune cells completely enclosed by tumor epithelial cells. [b]: The percentages of positive cells compared to the total amount of nucleated cells in the compartment. [c:] Mainly immune cells. LN+: Metastatic lymph nodes NS: Did not stain. NA: Not assessed

3.5 Determination of cut-off values

Categorization makes it easier to use information about the relationship between an outcome and a predictor variable. Many different approaches are used as cutoff strategies in explorative biomarker studies, whereof the most frequently used are mean/median, percentiles and systematic searches for the cutpoint associated with a minimum *P*-value (which best differentiates between outcome risk groups)[99,165].

3.5.1 Cut-offs and dichotomization

In Papers II and III, we explored the prognostic impact of molecules of which there exists no consensus with regard to expression in tumor epithelial or stromal/immune cells. For PD-L1 expression in tumor epithelial cells, antibodies to be used for prediction of treatment effect have been approved and implemented in routine clinical IHC, but is still a matter of debate.

We chose to analyze the prognostic impact and dichotomize patients according to the mean value of the four (or two, if one core was missing) scores available for each patient. In Paper I, dichotomization was based on “maximum score”, defined as the single highest score of the four or two (if one core is missing) cores available. In a previous study, both maximum and average scores were assessed in a training set, and the maximum score approach resulted in an optimal significant prognostic impact for stromal CD8[106]. A preliminary investigation had found that the high score was comparable with the score found in corresponding whole sections. Because we planned to evaluate the combined prognostic impact of CD45RO and CD8 in Paper I, we applied the maximum score approach. In fact, both approaches were explored in all three studies, producing comparable results with regard to prognostic impact.

3.5.2 Discussion: Determination of cutoff values

Different scoring approaches and cutoff strategies have different strengths and weaknesses. Depending on study design, the importance of false negative and false positive results is weighted differently. Studies applying a cutoff near mean values lowers the probability of type 1 errors (false positive), and can be more reproducible, but this is not necessarily the biologically correct threshold, resulting in increased type 2 error (false negatives).

The use of a continuous scale, e.g. by absolute count, reduces the loss of information which is introduced by dichotomizing, and allows more flexible analyses of the biomarkers, but is generally more time-consuming and less applicable in clinical decision-making.

Utilizing the data-dependent "optimal" cut point approach may result in many different optimal cutoffs, and makes comparison of biomarkers across studies difficult. The optimal p-value will differ within subpopulations of the cohort included, and the type 1 error rate can be high due mainly to multiple comparisons[165,166]. The minimum P-value strategy should be seen as a hypothesis-generating approach, and must subsequently be validated in independent studies.

In tumors where intratumoral heterogeneity is marked, a maximum score approach may underrepresent areas of low/weak expression, better reflected using mean or median scores. But by sampling at least two cores at different sites (stereology), heterogeneity is to a large extent taken into account. Also, low inter-core heterogeneity was found when assessed by ICC (Paper II).

3.6 Statistical analyses

Sample size was estimated with disease-specific survival (DSS) as the primary endpoint. To detect a hazard ratio change of 0.5 (a 50% reduction in event rate) associated with the presence of a tested biomarker (what represents a clinically significant effect), assuming that the 5-year DSS for stage I-IIIA NSCLC patients is around 60%, and the frequency of the biomarker is around 35%, a power of 80% at an alpha of 5% requires the inclusion of 300 subjects (PASS 2002, Number Cruncher Statistical Systems, Kaysville, Utah, USA) [167]. The estimate does not take into account the testing of multiple markers in the actual analysis, and can only serve as a rough indication of the number of needed subjects[167]. When performing update of endpoints in 2013, we decided to increase the sample size from 335 patients by including patients treated from 2005-2010. This would permit us to use a test- and validation set tom improve robustness and generalizability.

The statistical analyses were performed using the SPSS statistical package, version 22.0 (SPSS, Chicago, IL, USA).

The primary endpoints used were DSS, defined as the time from surgery to lung cancer death. Secondary endpoints were disease-free survival (DFS) and overall survival (OS), defined as the time from surgery to first lung cancer recurrence and to death of any cause, respectively.

Inter-rater reliability was assessed by comparing IHC scores from each observer by use of a to-way random effects model with absolute agreement, average-measures ICC (intraclass correlation coefficient) and Cohen's kappa, to assess the degree that scorers provided consistency in their ratings across cores. ICC was moderate or excellent (r=0.642-0.951), suggesting that scores were rated similarly across coders and a minimal amount of measurement error was introduced by the coders.

Statistical associations between molecular marker expressions and the clinicopathological variables was assessed by the χ^2 or Fischer exact test. Denoted r, the strength and direction of associations between molecular marker expressions was examined using Spearman's rank-correlation.

The Kaplan-Meier method was used to perform univariate analyses of survival according to each of the clinicopathological variables and molecular marker variables, and statistically significant differences between survival curves were assessed by the log-rank test. In addition, a Cox proportional hazards regression model with a single explanatory variable provided hazard ratios with

95% confidence intervals. Kaplan-Meyer curves were terminated at 120 months, a point-of time when approximately <10% of patients were at risk.

To examine the independent value of each molecular marker on outcome, they were included in multivariate analysis by use of the Cox Proportional Hazards model. All clinicopathological covariates found to be significant and assessed as important in the univariate analyses were included in an initial multivariate analysis (assessed separately for all patients, SCC and ADC histological subgroups, and N+ patient subgroup). Subsequently, the covariates found significant in the initial multivariate analysis were included together with the studied biomarker(s). The backward conditional method was used for model fitting, and probability for stepwise entry and removal was set at .05 and .10, respectively. For all analyses, two-sided *P*-values <0.05 were considered statistically significant. Proportional hazard assumptions were tested graphically (log-minus-log plot).

Adjustments for multiple testing are required in confirmatory, clinical studies whenever results from multiple tests have to be combined in one final conclusion and decision. But for exploratory studies, arguments against multiple test are not considered strictly necessary[168].

3.7 Ethics

The study was performed in accordance with the Helsinki Declaration of 1975, and was approved by The Norwegian Data Protection Authority and the Regional Committee for Medical and Health Research Ethics approved the study (Protocol ID: 2011/2503)[169].

The need for patient consent was waived, on the basis of: 1) a majority of patients were deceased at the time of inclusion (400/536, 75%); 2) retrospectively collecting informed consent from living patients or relatives of deceased patients would be costly with respect to time and money, might be considered a burden/inconvenience, and obtaining a valid consent could be challenging due to the high mean age at diagnosis; 3) that the risk of being included and disadvantage/breach of personal integrity caused by not being informed, can be considered minimal/vanishingly small; 4) the benefit for living (included) and future patients, relatives and society is potentially large. For future expansions of the study, a personal letter will be sent to all living included patients, so as they may actively exempt themselves from participating in the study.

The reporting of clinicopathological variables, survival data, and biomarker expressions was conducted in accordance with the REMARK guidelines[170].

4 MAIN RESULTS

4.1 Patient characteristics

Demographic, clinical and histopathological variables for all 536 patients and SCC and ADC histological subgroups, and their association with DSS is presented in Table 2, Chapter 3.

Of the 536 resected NSCLC patients included, 261 (49%) were diagnosed with a recurrence and 400 (75%) died, whereof 219 (55%) died from lung cancer during the follow-up period. Median DSS, DFS and OS was 127, 74 and 47 months, and respective 5-year survival was 58%, 55% and 45%. 285 (53%) patients were treated at the University Hospital of North Norway (UNN), Tromso, and 251 (47%) at Nordland Hospital (NH). There were no significant differences in DSS, DFS and OS between centers.

Median age was 67 (range 28-85) years. The majority of patients were men (68%), but the rate of women increased from 21% (1990-1995) to 44% (2006-2010).

The tumors comprised 289 (54%) SCCs, 201 (38%) ADCs and 46 (9%) large-cell carcinomas. The proportion of squamous cell carcinoma (SCC) decreased from 66% (1990-1995) to 47% (2006-2011) whereas adenocarcinoma (ADC) increased from 28% to 46%, a tendency seen in both sexes.

Seventy-six patients (14%) received adjuvant radiotherapy and 113 (21%) palliative radiotherapy. In Norway, adjuvant chemotherapy has been recommended to pathological stage (pStage) IB-IIIA patients with adequate performance status and no contraindications since 2002, limited to pStage II-IIIA from 2005. From 2005 to 2010, 43 stage II-IIIA patients received adjuvant chemotherapy, while 72 did not (1990-2004: data not registered).

Uni- and multivariate prognostic impacts of immunological markers assessed in this thesis on DSS are summarized in Table 7.

Table 7 Uni- and multivariate prognostic impacts of immunological markers on disease-specific survival (DSS) in all patients and stratified by histology.

	UNIVARIATE ANALYSES		MULTIVARIATE ANALYSESc	
	All patients	**SCC / ADC**	**All patients**	**SCC/ADC**
PRIMARY TUMOR				
Tumor epithelial cells				
T-CD45RO	Pos. (HR 1.62, *P*=0.007)	SCC: Pos. (HR 2.34, *P*=0.0003)	Pos. (HR 1.80, *P*=0.001)	SCC: Pos. (HR 2.65, *P*<0.001)
		ADC: ns		ADC: NE
T-PD-L1	ns	SCC: Pos. (HR:1.79, *P*=0.037)	NE	SCC: ns
		ADC: ns		ADC: NE
T-CTLA-4	ns	SCC: ns	NE	SCC: NE
		ADC: Pos. (HR 0.64, *P*=0.037)		ADC: ns
Stromal cells				
S-CD45RO	ns (HR 1.31, *P*= 0.050)	SCC: Pos. (HR 1.67, *P*=0.012)	NE	SCC: Pos. (HR 1.85, *P*=0.003)
		ADC: ns		ADC: NE
S-CD8	Pos. (HR 1.70, *P*=0.0002)	SCC: Pos. (HR 2.17, *P*=0.0002)	Pos. (HR 1.54, *P*=0.004)[d]	SCC: Pos. (HR 1.93, *P*=0.004)[d]
		ADC: ns		ADC: NE
S-PD-L1	Pos. (HR 1.55, *P*=0.004)	SCC: Pos. (HR 2.09, *P*=0.002)	Pos. (HR 1.47, *P*=0.014)	SCC: Pos. (HR 2.16, *P*=0.001)
		ADC: ns		ADC: NE
S-PD-1	ns	SCC: ns	NE	SCC: NE
		ADC: ns		ADC: NE
S-CTLA-4	ns	SCC: Pos. (HR 0.60, *P*=0.013	NE	SCC: Pos. (HR 0.62, *P*=0.021)
		ADC: ns		ADC: NE
Intraepithelial immune cells				
T-PD-1	Pos. (HR 1.42, *P*=0.012)	SCC: Pos. (HR 1.55, *P*=0.034)	Pos. (HR 1.48, *P*=0.005)	SCC: Pos. (HR 1.71, *P*=0.011)
		ADC: ns		ADC: NE
Combinations				
T-CD45R0+ S-CD8[a]	Pos. (HR 2.36, *P*=0.0001)	SCC: Pos. (HR 4.77, *P*<0.0001)	Pos. (HR 2.43, *P*=0.001)	SCC: Pos. (HR 6.50, *P*<0.001)
		ADC: ns		ADC: NE
S-PD-L1 + T-PD-1[b]	Pos. (HR 1.81, *P*<0.001)	SCC: Pos. (HR 2.06, *P*<0.001)	Pos. (HR 1.72, *P*<0.001)	SCC: Pos. (HR 2.14, *P*<0.001)
		ADC: Pos. (HR 1.52, *P*=0.045)		ADC: Pos. (HR 1.52, *P*=0.049)
METASTATIC LYMPH NODES				
Tumor epithelial cells				
T-PD-L1	ns	SCC: ns	NE	NE
		ADC: ns		
T-CTLA-4	Neg. (HR 1.65, *P*=0.037)	SCC: ns	Neg. (HR 1.65, *P*=0.039)	NE
		ADC: ns		
Intraepithelial immune cells				
T-PD-1	ns	SCC: ns	NE	NE
		ADC: ns		

[a]: High+ high versus low+low. [b]: Others versus low + low. [c]: In separate models, except for [d]: adjusted for T-CD45RO and PD-Immunoscore. All clinicopathological covariates significant in multivariate analysis are included in each model. Abbreviations: ADC, adenocarcinoma. HR, hazard ratio. N, number. N+ LN, metastatic lymph node. NE: not entered. NR, not reached. S, stroma. SCC, squamous cell carcinoma. T tumor.

4.2 Paper I

Based on the colorectal TNM-Immunoscore research, we wanted to explore CD45RO as a candidate for an NSCLC Immunoscore. We aimed to investigate the prognostic impact of the density of CD45RO+ T memory lymphocytes in the stromal and intraepithelial compartment of primary tumors in 536 stage I-IIIA NSCLC patients, and to assess its potential in a NSCLC Immunoscore, compared to or combined with previously published stromal CD8-analyses (S-CD8)[106]. Antibodies used were well-validated and used in the routine clinical and research.

4.2.1 Correlations

Extensive highly significant correlations between the two markers were found. The correlations observed were between maximum CD45RO-score in the two compartments (r=0.494), and between stromal CD45RO and CD8 scores (r=0.559). No significant associations between density of CD45RO and clinicopathological characteristics were found.

4.2.2 Univariate analyses

Low CD45RO density was a negative prognostic factor for DSS (HR 1.62, P=0.007), DFS and OS when scored in the intraepithelial compartment (T-CD45RO), and borderline significant in the stromal compartment (S-CD45RO, DSS: HR 1.31, P=0.050). In both compartments, low CD45RO was a significant negative prognostic factor for patients with tumors SCC histology (DSS: HR 2.34, P=0.0003 and HR 1.67, P=0.012, respectively) and not for ADC.

We then assessed the combination of T-CD45RO and CD8 in the stromal compartment (S-CD8), the most significant settings for each of the two immune markers. The combination of T-CD45RO and S-CD8 yielded substantially improved stratification of patients with regard to prognosis for DSS, DFS and OS, limited to the SCC subgroup (High + High versus Low + Low scores, DSS: HR 2.36, P=0.0001), significant across pathological stages I, II and IIIA (DSS: I: P=0.025; II: P<0.001, IIIA: P=0.001). The combination facilitated the identification of 5-year DSS differences between patients with High + High and Low + Low scores of 40%, 73% and 33% for pStage I, II and IIIA patients, respectively.

4.2.3 Multivariate analysis

A low CD45RO score in the intraepithelial compartment was an independent adverse prognosticator of DSS (HR 1.80, *P*=0.001), DFS and OS, in the entire cohort and for SCC patients (DSS: HR 2.65, *P*<0.001). For SCC patients only, stromal CD45RO score was also a positive prognosticator in multivariate analyses, but with a lower effect size (HR 1.85) and higher *P*-value (0.003). The combination of T-CD45RO and S-CD8 scores had an increased prognostic impact for DSS, DFS and OS in the whole cohort (DSS: High + High versus Low + Low: HR 2.43, *P*=0.001) and the SCC subgroup (DSS: HR 6.50*, P*<0.001), significant within each pathological stage (pStage I: HR 4.35, II: HR 8.24, IIIA: 9.52, all *P*≤0.012) and in both centers (UNN: HR 4.82, NH: HR 5.94, both *P*≤0.001).

4.3 Paper II

PD-L1 has recently been introduced as a predictive biomarker for anti-PD-1 treatment, but the prognostic impact of PD-L1 and PD-1 in NSCLC is debated. We aimed to explore the expression of PD-L1 and PD-1 in the tumor epithelial and stromal compartments of 536 primary tumors and 142 corresponding metastatic lymph nodes, in order to evaluate their prognostic impact and potential as NSCLC-Immunoscore candidates. Several antibodies were subject to thorough validation before final analyses were performed.

4.3.1 Expression and correlations

PD-L1 staining was cytoplasmatic +/- membranous in tumor epithelial cells, and was scored according to intensity (T-PD-L1). In the stromal compartment, the density of PD-L1+ and PD-1+ cells was scored (S-PD-1, S-PD-L1), and the density of PD-1+ immune cells was also scored in the intraepithelial compartment (T-PD-1). Neither PD-L1 nor PD-1 were associated with clinicopathological variables such as histological subgroup or pathological stage.

We found a low level of heterogeneity between the two cores sampled from each tumor (ICC 0.726-0.844) and excellent between-scorer agreement (ICC>0.830). We observed extensive correlations between mean scores of PD-L1 and PD-1 in primary tumors, but not between expression in primary tumors and lymph nodes. T-PD-1 was significantly lower in metastatic LN (30%) than in primary tumors (56%) (P<0.001).

4.3.2 Univariate analyses

Low density of S-PD-L1 (HR: 1.55, *P*=0.004) and of T-PD-1 (HR 1.42, P=0.012) was associated with poor DSS, limited to the SCC subgroup (S-PD-L1: HR 2.09, *P*=0.002; T-PD-1: HR 1.55, *P*=0.034). Low tumor epithelial PD-L1 was also a negative prognostic factor for SCC patients (HR 1.79, P=0.037) in univariate analyses.

The combination of the two variables of strongest prognostic impact in the entire cohort, low S-PD-L1 and low T-PD-1 (“PD-Immunoscore”), was a significant negative prognostic factor for DSS in all patients (HR 1.81, *P*<0.001) in all pathological stages (I, II, IIIA), and it had a strong prognostic

impact for SCC patients (HR: 2.06, *P*<0.001) in both centers (UNN, NH) and all endpoints (DSS, DFS, OS). The expression of PD-L1 and PD-1 in LN metastases was not associated with outcome.

4.3.3 Multivariate analysis

Low expression of S-PD-L1 and of T-PD-1 were both independent negative prognostic factors for DSS (HR 1.41, *P*=0.031; HR 1.39, *P*=0.025, respectively, when included in the same multivariate model). The combination of low scores (PD-Immunoscore) was an independent prognosticator for poor survival for DSS, DFS and OS (HR 1.72, 1.57, 1.36, respectively, all *P*<0.001). Histological subgroup analyses showed that the PD-Immunoscore was a significant independent prognostic factor mainly for the SCC subgroup (SCC: HR 2.14, *P*<0.001; ADC: HR 1.52, P=0.049). Adjustment for S-CD8 and T-CD45RO weakened the prognostic impact of PD-Immunoscore (DSS: HR 1.48, *P*=0.010), but did not eliminate it.

4.4 Paper III

CTLA-4 is frequently overexpressed in cancers, and therapeutic blockade has shown some efficacy in NSCLC, but its prognostic impact in NSCLC is unclear. We aimed to explore the expression and prognostic impact of CTLA-4 in the stromal and tumor epithelial compartments of 536 primary tumors (PT) and 142 corresponding LN metastases, and to evaluate its potential as candidate for a NSCLC Immunoscore. Thorough in-house validation of antibodies was performed.

4.4.1 Expression and correlations

CTLA-4 cytoplasmatic +/- membranous staining was relatively homogenously distributed in both tumor epithelial cells (T-CTLA-4) and stromal immune cells, and intensity was scored. Scores in the two compartments of PTs were significantly correlated with each other (r=0.329) and with CD3, CD4, CD8, CD45RO, CD20, PD-1 and PD-L1. Mean CTLA-4 scores in tumor epithelium of PTs and metastatic LN did not correlate, and were discordant in 44% of cases.

No significant associations between clinicopathological variables and CTLA-4 in PT were found. High T-CTLA-4 was more common in metastatic LN of patients with poorly differentiated PTs (P=0.034), and was strongly correlated to T-PD-L1 score (r=0.404). Between-scorer agreement was excellent (ICC>0.882).

4.4.2 Univariate analyses

For all PTs, expression of CTLA-4 did not significantly predict DSS. In subgroup analyses, high stromal CTLA-4 was a positive prognostic factor for DSS in SCC patients (HR 0.60, P=0.013) and tumor epithelial CTLA-4 for ADC patients (HR 0.64, P=0.037). Combinations with other, previously evaluated, immunological markers did not contribute to substantial improved prognostic impact compared to single markers. Tumor epithelial CTLA-4 in metastatic LN was associated with poor DSS (HR 1.65, P=0.037).

4.4.3 Multivariate analysis

In primary tumors, high stromal CTLA-4 was an independent positive prognostic factor (HR 0.62, *P*=0.021) for SCC patients. Tumor epithelial CTLA-4 expression was not an independent prognostic factor in primary tumors, but had an independent negative prognostic impact in metastatic LN (HR 1.65, *P*=0.039).

5 GENERAL DISCUSSION

5.1 Summary of strengths and weaknesses

Strengths and weaknesses of the studies presented in this paper have been discussed in detail in chapter 3, Materials and methods. A summary of strengths and weaknesses are presented in Table 8.

Table 8. A summary of strengths and weaknesses

Weaknesses	Strengths
Study design and database	
No validation of results in external patient cohorts	Large cohort and extensive follow-up
Long inclusion period, potential differences in treatment over time may confound results	Minimal differences treatment at each point in time, due to centralized and standardized surgical treatment
Information bias due to retrospective collection of data (treatment, recurrence and cause of death)	Minimal selection bias due to inclusion of consecutive patients and centralized treatment.
nadequate data with regard to mutational status (EGFR, ALK, ROS1) and smoking.	Data from medical records and pathological archives and follow-up from local hospitals or GPs collected by clinician optimizes quality of database, especially with regard to endpoints. No patients lost to follow-up.
Limited N+ sample size increasing risk of type 2 error in corresponding analyses	All tumors staged according to most recent classification, by an expert pathologist
	Inclusion of both PT and corresponding metastatic LN broadens research focus
Tissue microarray and immunohistochemistry	
Assembly and IHC analyses demand time and requires technical skill and methodical approach at assembly	Well-validated and high through-put method which saves time, tissue, reagents and money
Cores from central tumor and invasive margin not prespecified for inclusion	Cores from both stromal and neoplastic areas included in TMA especially important for immune biomarkers
Size and number of cores, and potential heterogenous biomarker expression may reduce representativity and increase risk of type 2 error	Impact of heterogeneous tumors is taken into account by stereology and selection of representative areas for sampling, confirmed by relatively high scoring agreement between cores
Variability introduced by preanalytic factors (e.g. fixation), experimental conditions (e.g. staining protocol) and antigen quality	Maximal standardization of analysis
Potentially high intra- and interobserver variability	Subjective interpretation improved compared to WTS
Monoclonal antibodies used are more prone to false negative results (type 2 error)	Antibodies used were well-validated and in routine use or carefully validated by us
Scoring and data analyses	
Manual, semi-quantitative scoring difficult to reproduce and compare between studies	Semi-quantitative scoring is simple, low-cost, quick, transferrable into clinical practice
Ordinal variables have more information loss than continuous variables	Scores from two independent scorers, blinded from outcome and other variables, were included and inter-rater agreement was good or excellent
Minimum *P*-value has increased type 1 error (false positive) and difficult to compare across studies	Minimum *P*-value approach appropriate for exploratory studies (reduced type 2 error), several significant threshold values observed

5.2 Paper I

Our study demonstrated a positive prognostic impact of CD45RO+ TILs for SCC patients, especially when quantified in the intraepithelial compartment. The prognostic impact was strong (HR 2.65) and robust: independent of pathological stage and confirmed in both centers and for DSS, DFS and OS. The presence of activated effector memory T cells are considered to be manifestations of a tumor-specific host immune response associated with cytotoxicity and a tumor-suppressive T_H1 orientation[62].

A high tumor infiltration of memory T cells expressing the CD45RO marker measured by IHC has been demonstrated to be a positive prognostic factor for most solid cancer types reported, hence, the positive impact in SCC patients was not surprising[171]. However, inconsistencies in expression levels and varying study design (quantification methods, antibodies, localization, pStage, cutoff and outcome) limit comparability between studies. Moreover, the strength of the association between memory T cells and outcome differs between cancer types. Also, some studies have found the prognostic impact to vary depending factors such as pathological stage[109,115,117,172]. In renal cell carcinoma (RCC), increased CD45RO+ TIL infiltration is associated with adverse survival, illustrating that the presence of T memory cells may also play a part in, or be overcome by, an immunosuppressive TME in tumors[117].

We, and others, found no significant association between CD45RO+ TILs and outcome for ADC patients[120]. Differences between SCC and ADC subgroups in NSCLC include clinicopatholgical features (e.g. smoking history, oncogenic mutations, prognosis) and treatment response and toxicity, supporting that the two are distinct NSCLC entities[173]. Although the prevalence of CD45RO+ (or CD8+ TILs) in SCC and ADC primary tumors was similar, the prognostic impacts diverged. This is consistent with differences in the TME, potentially explained by differing functional orientation or activation status, rather than their density. Alternatively, the impact of tumor-cell intrinsic traits might dominate over that of the TME in ADC tumors ADC, but not SCC histology.

Assessing the density of CD45RO and CD8 in primary tumors in a combined score, similar to the Immunoscore approach in CRC, predicted SCC patient outcomes better than single markers independent of TNM stage. Recently, our group studied the prognostic impact of intraepithelial CD3+, CD4+, CD8+, CD20+ and CD45RO+ TILs in metastatic lymph nodes from the N+ subgroup of the same cohort[174]. Parallel to our observations in primary tumors, we observed that a high CD45RO score in

metastatic LN was an independent positive prognostic factor for DSS, limited to the SCC subgroup of N+ patients (HR 0.31, *P*=0.003). A prognostic impact reproducible from primary to metastatic tumors has previously been demonstrated in CRC and RCC patients[175]. Strikingly, CD45RO was the only one of the immune markers included in our study that showed a prognostic impact when expressed in metastatic LN. To our knowledge, no other studies assessing CD45RO protein expression in NSCLC have been published since the publication of Paper I.

A significant proportion of NSCLC patients who are surgically treated with curative intent, experience recurrences, whereof most succumb to the disease, even when diagnosed with early stage disease (Table 2). Our results imply that a high density of effector memory T cells contributes to the prevention of recurrence, metastases and death from stage I-IIIA NSCLC, or is a surrogate marker for other factors that do. Quantification of CD45RO and CD8 may serve as a clinically useful supplement to the TNM classification, but must be validated in independent patient cohorts, preferably in prospective studies with predefined scoring cutoffs.

5.3 Paper II

We demonstrated that PD-L1 expression was comparable in SCC and ADC, more commonly on immune cells than tumor epithelial cells. We found that a low density of stromal PD-L1+ immune cells and of PD-1+ TILs infiltrating the tumor epithelial compartment of primary tumors both independently predicted a negative outcome, confirmed in two centers and all pathological stages. The prognostic impact was present in the SCC, but not ADC, histological subgroup. When assessed in the same multivariate model, stromal PD-L1 score had the greater impact. Low scores for both markers ("PD-Immunoscore") significantly predicted adverse outcomes in uni- and multivariate analyses for DSS, DFS and OS, and was an independent significant prognostic factor, also when adjusted for other immunological markers of prognostic importance in the same patient cohort, T-CD45RO and S-CD8. No prognostic effect of PD-1 or PD-L1 was seen in lymph node metastases.

The PD-1 pathway has emerged as an essential immune regulatory mechanism in cancer, and exploring its regulatory mechanisms, prognostic significance and potential in cancer treatment has become a top priority in cancer research[176]. Expression of PD-L1 has been shown in several malignancies, including NSCLC, in tumor cells as well as immune cells of lymphoid and myeloid lineage[177]. Early reports suggested that overexpression of PD-L1 in tumor cells was a tumor-intrinsic mechanism, that it was immunosuppressive and inversely correlated with TILs, associated with poor prognosis[178]. Later reports demonstrated that PD-L1 can be induced in tumor and immune cells by INFγ and other cytokines released in an inflammatory TME, and that it was strongly associated with the presence of TILs. Resulting in suppression of local effector T cell function, it was seen as an adaptive immune-resistance mechanism[179]. The expression of PD-L1 in tumor cells is thus influenced both by intrinsic and extrinsic regulation, and may display heterogeneous and dynamic expression. The regulation of PD-1 expression is also complex. A transient increase in expression is seen as part of the normal regulation of T cells, but it is also one of several markers, of which the cumulative expression is associated with T cell exhaustion/dysfunction[180].

PD-1 blockade can restore T cell function leading to enhanced anti-tumor immune responses in several cancers, including advanced melanoma, NSCLC, RCC, bladder cancer and Hodgkin lymphoma[181]. Clinical trials have shown impressive responses in advanced NSCLC, and patients experience a lower frequency of adverse events compared to chemotherapy[21]. Hopes are high that immunotherapy will displace chemotherapy, on basis of investigations of several monoclonal

antibodies targeting both PD-1 and PD-L1 in the 1st, 2nd and adjuvant setting, and combinations with chemotherapy, immunotherapy, radiotherapy[182].

Response to PD-1 blockade is limited to a fraction of patients (approximately 20% in unselected patients), with sustained response in a subset of these, prompting a search for factors predicting therapeutic response[183]. Currently the most studied candidate, the expression of PD-L1 in tumor cells has been found predictive of response in several clinical trial[183]. A majority of studies show an improved ORR, OS and PFS in patients with higher tumor PD-L1 level, but patients with low or negative levels of PD-L1 also may respond to the treatment, introducing a challenge for clinicians[182]. Adding to the complexity, PD-L1 expression on tumor-infiltrating immune cells may also predict response, independently from tumor epithelial PD-L1 expression[47,185]. The predictive value of PD-L1 is still object of debate because of a diversity of antibody clones, staining platforms and scoring criteria, potential tumor heterogeneity and challenges regarding access to adequate tissue[183].

Similarly, the role of PD-L1 expression as a prognostic marker in NSCLC has been widely reported, but is challenged by a diversity of method and inconsistent results. However, few others have explored the prognostic impact of PD-1-pathway molecules when expressed in immune cells, or comparing intraepithelial and stromal compartments[186–188]. Since the completion of Paper II, several studies have reported the prognostic value of tumor cell PD-L1 expression in early stage NSCLC patients, reporting positive, none or poor prognosis in different subgroups[189–195]. Methods of analysis and scoring and expression levels vary considerably. One large study demonstrated an association between tumor PD-L1 and lymphocyte infiltration with CD3+ and CD8+ positive immune cells, consistent with induction by Th1/IFNγ-singaling[193]. In line with this, we found T-PD-L1 to be correlated with stromal CD3 (r=0.391) and CD8 (r=0.345) (not previously published).

In our study, the combination of high stromal PD-L1+ immune cells and intratumoral PD-1+ TILs was associated with a favorable prognosis, even though the PD-1/PD-L1 pathway is recognized as a negative regulator of T cell activation. We suggest that this may explained by a positive net impact of activated immune cells triggering negative feedback mechanisms which induce upregulation of PD-L1. In contrast, a low PD-Immunoscore may represent a TME which lacks inflammatory mediators, because of a predominance of immunosuppressive cell types and/or non-functional infiltrating anti-tumor immune cells. Our study did not reveal reasons for the difference in prognostic impact of PD-1/PD-L1 expression between SCC and ADC patients.

Interestingly, improved responses of PD-1 blockade are thought to be most effective in "inflamed tumors" characterized by pre-existing (CD8+) TIL infiltration and PD-L1 expression in the TME[179,196,197]. A classification of tumors into four different types, based on the presence or absence of (CD8+) TILs and PD-L1 expression, has been suggested as an initial method to identify which pathways are driving tumor progression. These include type I: adaptive resistance (PD-L1+, TIL-), type II: immunological ignorance (PD-L1-, TIL-), type III: intrinsic induction (PD-L1+, TIL-) and type IV: immunological tolerance (PD-L1-, TIL+)[179,198,199]. Type I is frequent in lung cancer, and was recently reported to be associated with a high mutational burden/neoantigens and PD-L1 amplification, hence expected to have a favorable outcome in response to PD-1 blockade[199]. The study by Ock et al. also suggests that assessment of TILs together with PD-L1 improves prognostic stratification of patients. The PD-Immunoscore may in a similar way reflect the importance of an activated immune infiltrate for prognosis, and would be interesting to investigate as predictor of response to immunotherapy.

5.4 Paper III

Except for the successful improvement of overall survival in studies involving patients with advanced melanoma, only modest clinical benefits have been observed with CTLA-4 antibody therapy in NSCLC. CTLA-4 blockade is not FDA-approved for NSCLC patients, but studies are ongoing, including combinations with other immunotherapies[200]. In contrast to the volume of reports concerning PD-1 pathway molecules, few have reported the prognostic impact of CTLA-4 protein expression in different tumors, and results are conflicting[201–207]. One study has reported a lack of association between tumor cell CTLA-4 expression and NSCLC survival, similar to our results[205]. Other studies assessing CTLA-4 in the stromal compartment are missing.

The main finding in Paper III was that high CTLA-4 expression in tumor epithelial cells located in regional lymph nodes is associated with independent negative prognostic impact. Interestingly, CTLA-4 expression in primary tumors did not predict outcome, and was not correlated with expression in lymph node tumor cells. We suggest that this discordance is related to genetic heterogeneity between primary and metastatic tumors, not correlated to the status of the immune system in primary tumors, but potentially an immunosuppressed TME in lymph nodes. Because of a limited number of evaluable metastatic lymph nodes and relatively high p-values, results must be interpreted with caution. The study was not powered to look at differences between SCC and ADC subgroups for N+ patients.

Contrary to this finding, high CTLA-4 in stromal cells of predominant immune cell morphology was an independent predictor of positive DSS for SCC patients. Similar to the results in Paper II, this may be interpreted as a positive effect of a TME which is generally immune activated. High stromal CTLA-4 may be associated with a balance between anti-tumor and pro-tumor immune properties tipped towards the former, supported by the correlation with other immune markers of positive prognostic value (stromal CD8 and PD-L1). Assessing immune cell expression of CTLA-4 by IHC as a predictor of CTLA-4-blockade or other immunotherapy would be most interesting. However, the broad expression of CTLA-4 in several stromal cell types indicates that an initial exploration of more specific antibodies for CTLA-4, or the use of multiplexed staining to discern immune cell subtypes, might be useful.

6 CONCLUSIONS AND IMPLICATIONS FOR FURTHER RESEARCH

Molecular features of the malignant cells have long been the focus of cancer research, but in recent years, pathological characterization of the immune TME has provided new insight. Multiple studies have demonstrated a correlation between the location, type and functional orientation of tumor infiltrating immune cells and cancer patient outcome. Pioneer work in colorectal cancer and reports indicating a prognostic importance of TILs in NSCLC, suggests that quantification of the immune TME may provide information supplementing the TNM-classification in predicting outcome for NSCLC patients, likely to influence therapeutic management. Presumably, patient prognoses are shaped both by attributes of neoplastic cells and the state of the local adaptive immune response, which must be taken into account to be able to accurately predict outcomes.

This thesis presents the results of three studies in which we have studied the protein expression of immunological markers, quantified by IHC in a large TMA material of early stage NSCLC tumors, and their association with patient outcome. Assessment of both tumor and stromal compartments, as well as primary tumors and corresponding metastatic lymph nodes, has provided enhanced detail of expression patterns and has shed light on the importance of the location of immune marker expression with regard to prognostic impact.

In the first study, we have identified a *robust and* independent positive prognostic impact of memory T cells infiltrating the tumor epithelium of patients with SCC tumor histology, but not for ADC tumors. This highlights distinctions between the two major NSCLC subgroups, possibly related to properties of TME immune cells. Quantifying the density of both CD45RO and CD8 in the TME of SCC tumors enabled an improved identification of patients with low- and high risk of poor outcome. As a result, a stratification of patients with considerable differences in 5-year survival within each pathological stage was possible*,* hence supplementing, but not outcompeting the TNM-classification. Consequently, this method of assessing effector/memory T cells is a potential candidate for inclusion in a NSCLC TNM-Immunoscore (TNM-I).

In fact, the clinical implementation of such an approach is about to be explored in a prospective multicenter study, initiated by our research group. In this study, we attempt to validate methods and improve reproducibility and reliability of promising candidate markers from explorative

studies, including markers presented in this thesis. If successfully established, a NSCLC TNM-I may have a major impact on treatment strategies for this patient group. Hopefully, we will be able to, more precisely, select patients with high risk of poor outcome (despite low pathological stage) for adjuvant therapy, and more adequately decide how aggressive adjuvant treatment should be. Importantly, we may avoid the inappropriate and costly treatment of postoperative chemotherapy for selected patients. Furthermore, we aim to establish TMN-I as a predictive tool in a phase II study in the adjuvant setting, in which immunotherapeutic drugs are likely to be included.
Finally, the positive prognostic impact of memory T cells may underpin research and development of therapy intended to stimulate generation and maintenance of such cells in the treatment of NSCLC; immune checkpoint inhibitors, agonists of co-stimulatory receptors on memory T cells, cancer vaccines activating or inducing memory T cells or adoptive T cell transfer of stem cell memory T cells.

The second paper concludes that the expression of PD-1 and PD-L1 in tumor infiltrating immune cells predicts survival independently from pathological stage. We believe that this reflects a positive influence of infiltration by activated immune cells in favor of the host anti-tumor response, rather than the adaptive tumor-response. However, a plethora of previous studies with heterogeneous methods and inconsistent conclusions illustrate that the biological mechanisms involved, and the potential prognostic and predictive implications of the PD-1 pathway molecules in NSCLC remain to be further elucidated. The many unanswered questions call for a more stringent validation of our findings before potential clinical implementation.

Tumor heterogeneity and representativity of samples is major challenge with regard to biomarker studies, and findings in our third study illustrate that immune markers sampled from primary tumors cells and metastatic cells may be discordant and of varying prognostic impact. The independent positive prognostic impact of expression of CTLA-4 in stromal cells may further strengthen arguments in Paper I and 2, that the presence of activated immune cells in NSCLC tumors is of prognostic importance. Even when immune markers represent negative regulators of T cell activation and function, they may function as surrogate markers for the presence of an activated and, to some extent, functioning anti-tumor host response.

Our research in a large data set with well-annotated samples and extensive follow-up will hopefully facilitate future contributions regarding prognostic, and possibly predictive, immune markers in NSCLC, eventually improving treatment strategies and outcome of this patient group.

Future exploration of immune markers in the context of smoking status, oncogene-driven mutation profiles, mutational burden/neoantigens and the microbiome might be of interest. A more detailed analysis of immune cells with regard to location, cytokine expression patterns, gene signatures and (other) immune checkpoints will hopefully contribute to even more efficient ways of predicting patient prognosis in the future.

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