UiT
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The prognostic impact of immune-related markers in non-small cell lung cancer

A retrospective study evaluating marker expression by immunohistochemistry on tissue microarrays

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A dissertation for the degree of Philosophiae Doctor.

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SUMMARY

Lung cancer is the leading cancer killer worldwide and non-small cell lung cancer (NSCLC) is the predominant subtype of lung cancer. The immune system plays an important role in cancer development. Immune-related markers expressed in the tumor microenvironment of resection specimens predict prognosis in many cancers, and may be potential targets for therapy.

We aimed to characterize the expression of selected-immune related markers in different cohorts of surgically resected NSCLC patients and relate their expression to prognosis. We constructed tissue microarrays and evaluated marker expression by immunohistochemistry.

In 55 patients treated with postoperative radiotherapy, increased expression of the T-cell markers CD4 and CD8 indicated was associated with a markedly improved disease-spesific survival.

We investigated the expression of the chemokines CXCR6 and CXCL16 in 335 NSCLC patients, and showed that lower CXCL16 expression by cells in the tumor stroma and in cancer cells was associated with worse disease-specific survival. In cell-based assays, we found that silencing CXCL16 expression in lung cancer cells increased proliferation.

We have previously identified stromal CD8 expression as a promising prognostic marker in NSCLC. Using a training cohort (*n* = 155) and three validation cohorts (*n = 169*, n = *295*, n = *178*), we found that increased stromal CD8 expression was consistently associated with improved survival. When the cohorts where combined, stromal CD8 was significantly associated with disease-specific and overall survival for all pathological stages, and may therefore be a good candidate marker for an NSCLC Immunoscore.

Lastly, we investigated the expression of the immune checkpoint LAG-3 in both primary NSCLC tumors (*n* = *553*) and their metastatic lymph nodes (*n* = 143). We found that increased expression of LAG-3 on tumor-infiltrating lymphocytes in both primary tumors and metastatic lymph nodes was associated with improved disease-specific survival.

Based on our present findings, we believe that evaluating immune-related markers can have an important prognostic utility in NSCLC. Validation of these findings in adequately powered prospective trials will be crucial for their potential clinical implementation.

LIST OF PAPERS

PAPER 1

Hald SM, Bremnes RM, Al-Shibli K, Al-Saad S, Andersen S, Stenvold H, Busund LT, Dønnem T. CD4/CD8 co-expression shows independent prognostic impact in resected non-small cell lung cancer patients treated with adjuvant radiotherapy. Lung Cancer 2013, 80(2); 209-215.

PAPER 2

Hald SM, Kiselev Y, Al-Saad S, Richardsen E, Johannessen C, Eilertsen M, Kilvaer TK, Al-Shibli K, Andersen S, Busund LT, Bremnes RM, Donnem T. Prognostic impact of CXCL16 and CXCR6 in non-small cell lung cancer: combined high CXCL16 expression in tumor stroma and cancer cells yields improved survival. BMC Cancer 2015, 15:441

PAPER 3

Donnem T, Hald SM, Paulsen E, Richadsen E, Al-Saad S, Kilvaer T, Brustugun T, Helland A, Lund-Iversen M, Poehl M, Olsen KE, Al-Saad K, Kiselev Y, Sandanger T, Andersen S, Pezzella F, Busund LT, Bremnes RM. Stromal CD8+ T-cell Density-A Promising Supplement to TNM Staging in Non-Small Cell Lung Cancer. Clinical Cancer Research, 2015, 21(11); 2635-43.

PAPER 4

Hald SM, Khanehkenari MR, Martinez I, Richardsen E, Al-Saad S, Kilvaer TK, Paulsen EE, Andersen S, Blix E, Busund LT, Bremnes RM, Donnem T. LAG-3 in non-small cell lung cancer: expression in primary tumors and metastatic lymph nodes is associated with improved surival.
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LIST OF ABBREVIATIONS

ADC	Adenocarcinoma
ALK	ALK receptor tyrosine kinase
APC	Antigen-presenting cell
BAC	Bronchioloalveolar carcinoma
BRAF	B-Raf proto-oncogene, serine/threonine kinase
CCL	C-C motif ligand
CCR	C-C motif receptor
CD	Cluster of differentiation
CK5	Cytokeratin
COPD	Chronic obstructive pulmonary disease
CT	Computed tomography
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
CXCL	C-C-motif ligand
CXCR	C-X-C motif receptor
DAB	3,3'-Diaminobenzidine
DDR2	Discoidin domain receptor tyrosine kinase 2
DFS	Disease-free survival
DNA	Deoxyribonucleic acid
DSS	Disease-specific survival
ECOG	Eastern Cooperative Oncology Group
EGFR	Epidermal growth factor receptor
FDA	The Food and Drug Administration
FGFR	Fibroblast growth factor receptor
FOXP3	Forkhead box P3
GM-CSF	Granulocyte-macrophage colony stimulating factor
H&E	Hematoxylin and eosin
HER2	Human epidermal growth factor receptor 2
HLA	Human leucoyte antigen
IASLC	International Association for the Study of Lung Cancer
IHC	Immunohistochemistry
IK-1B	IK cytokine, down-regulator of HLA II
KRAS	KRAS proto-oncogene, GTPase
LAG-3	Lymphocyte activation gene 3
LCC	Large cell carcinoma
MDSC	Myeloid-derived suppressor cell
MET	MET proto-oncogene, receptor tyrosine kinase
MHC	Major histocompatibility complex
n	Number
NH	Nordland Hospital
NK	Natural killer
NSCLC	Non-small cell lung cancer
OS	Overall survival
OUH	Odense University Hospital
OUS	Oslo University Hospital
PD-1	Programmed death receptor
PDGF	Platelet-derived growth factor
PDL-1	Programmed death-ligand-1
PET	Positron emission tomography

PGE2	Prostaglandin E2
PI3K	phosphatidylinositol-3 kinase
PORT	Postoperative radiotherapy
RNA	Ribonucleic acid
ROS1	ROS proto-oncogene 1, receptor tyrosine kinase
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
siRNA	small interfering RNA
TAA	Tumor associated antigen
TAP	Transporter associated with antigen processing
TGF-β	Transforming growth factor beta
Th	T helper cell subset
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TIL	Tumor-infiltrating lymphocyte
TIM-3	T cell immunoglobin and mucin-3
TLS	Tertiary lymphoid structure
TMA	Tissue microarray
TNM	Tumor, node, metastasis
Treg	Regulatory T-cells
TTF-1	Thyroid transcription factor-1
UICC	Union for International Cancer Control
UNN	University Hospital of North Norway
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

1. INTRODUCTION

1.1 Lung cancer

1.1.1 Epidemiology

Lung cancer is the most common cancer world-wide as well as the leading cause of cancer related mortality, causing approximately 1.6 million deaths in 2012 [1]. Estimations of cancer mortality in 2030 indicate that it will remain the number one cancer killer also in the foreseeable future [2]. In Norway, 1198 men and 960 women died of lung cancer in 2014, representing 20.5% and 18.7% of all cancer deaths, respectively. It is estimated that lung cancer resulted in a loss of 32 691 life years in Norway in 2012, approximately as many years as colon, breast and prostate cancer combined [3].

In men, lung cancer was the second most common cancer diagnosed in Norway 2015 (1564 new cases) after prostate cancer, while it for women was the third most common cancer (1471 new case) after breast and colon cancer [4]. While the incidence rates for lung cancer in men peaked in the early 1990s, the rates for women are still increasing. In the last five-year period, the rate of lung cancer for women in Norway was 10% higher than the previous period. In men the rate has declined slightly (Figure 1).

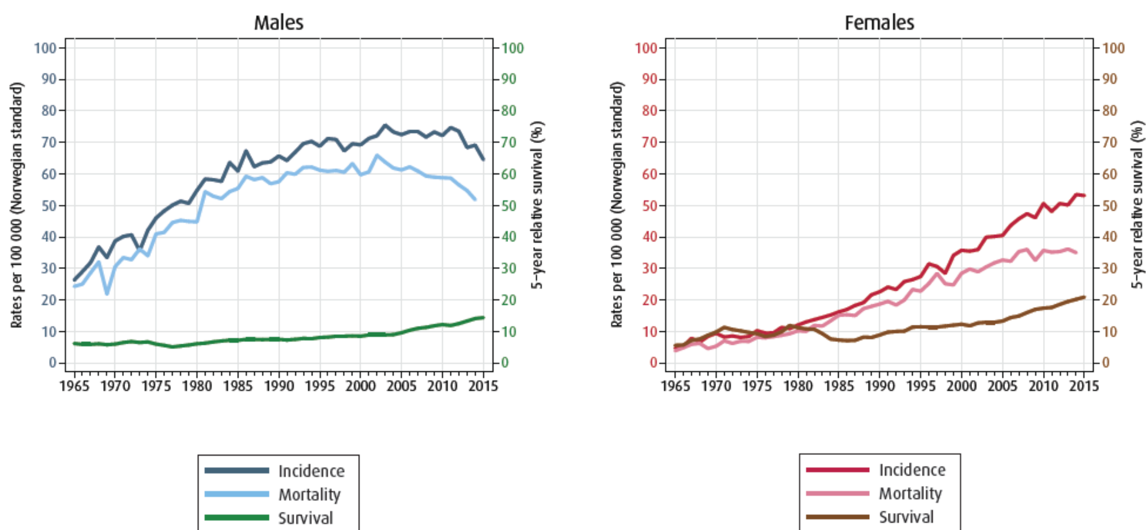

Figure 1: Trends in incidence and mortality rates and 5-year relative survival proportions (adapted from Cancer in Norway 2015 - Cancer incidence, mortality, survival and prevalence in Norway, 2016, Cancer Registry of Norway)

The main causative agent of lung cancer is smoking, and the difference in incidence rates between the sexes reflects historical differences in smoking patterns, with widespread smoking in women becoming common decades after it became common in men (Figure 2) [5].

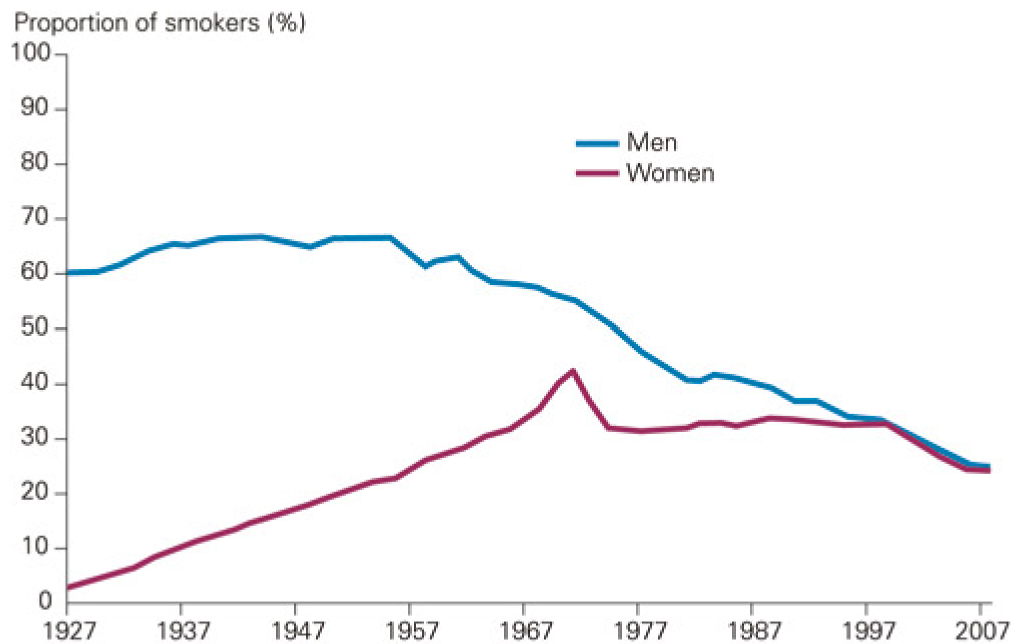

Figure 2: Proportion of smokers among men and women living in Norway in the period 1927 – 2007 (three-year averages). (Adapted from Lund, KE, Lund M, and Bryhni A, Tobacco consumption among men and women 1927-2007. Tidsskr Nor Laegeforen, 2009).

The incidence of lung cancer increases by age, with a median age at diagnosis of 71 years; approximately 70% of all new cases of lung cancer is diagnosed in patients 65 years or older [6]. The mortality rate of lung cancer closely follows the incidence rate, reflecting the fact that most patients diagnosed with lung cancer ultimately succumb to the disease.

The relative 5-year lung cancer overall survival (OS) for men in Norway was 14.4% between 2011 and 2015, an increase of 8.1 percentage points from the period 1976-1980 (6.1 % OS). Similarly, the 5-year relative OS for women was 20.9 % between 2011 and 2015, an increase of 9.7 percentage points from 1976-1980 (11.2 % OS).

1.1.2 Etiology

The link between smoking and lung cancer is one of the most thoroughly documented causal relationships in modern medicine [7]. The incidence of lung cancer closely follows the smoking epidemic, with a latency of around 20 to 30 years [8]. The risk of developing lung cancer increases in relation to the amount of cigarettes smoked daily and the number of spent years smoking [7]. Approximately 85% of all cases of lung cancer are caused by smoking [9]. However, if regarded as a separate cancer, lung cancer in never smokers would rank as the seventh most common cause of

cancer deaths worldwide [10]. In addition to smoking, known or suspected causes of lung cancer include exposure to radon, asbestos, indoor and outdoor pollution as well as genetic factors [10-13].

1.1.3 Histopathology

Lung cancer is divided into two major categories, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), comprising approximately 10-15 % and 85-90 % of all lung cancer respectively [14]. NSCLC is further divided into three main histological types: squamous cell carcinoma (SCC), adenocarcinoma (ADC) and large cell carcinoma (LCC) [15], with ADC and SCC the predominant types. SCC typically arises from bronchial epithelium in the larger proximal bronchi, though it can also occur more peripherally [16], whereas ADC commonly arise peripherally [17]. Immunohistochemistry may aide the pathologic classification of lung cancer, ADC is typically positive for the markers TTF-1/CK7, while SCC display positivity for p64/p40 and CK5/6. The diagnosis of LCC is reserved for NSCLC tumors without histological or immunohistological characteristics of ADC or SCC and now accounts for approximately 3 % of all lung cancer cases, though historically its rates have been higher [17]. The most recent edition of the World Health Organization (WHO) classification of lung tumors was published in 2015 [18]. Important changes from the previous 2004 editions include an increased emphasis on the use of immunohistochemistry on resection specimens and inclusion of a new classification for adenocarcinomas.

The relative frequencies of NSCLC subtypes has changed during the latest decades, with the incidence of SCC falling and that of ADC rising, currently making ADC the most common form of NSCLC [19]. Increasing use of filter cigarettes, deeper inhalation, as well as changes to the relative proportions of different carcinogens in cigarette smoke has been suggested as possible causes for this change [20,21].

Historically the most important demarcation in lung cancer is that between NSCLC and SCLC, as they differ markedly in growth rate, ability to metastasize and sensitivity to chemotherapy [22]. However, the last decade has seen a renewed emphasis on histology within the NSLSC group, as therapies such as the anti-folate agent pemetrexed and the angiogenesis inhibitor bevacizumab are only effective for non-SCC tumors [23,24].

1.1.4 Molecular genetics

While driver-mutations such as KRAS have long been recognized in NSCLC [25], the revolution in sequencing technology during the last decades has allowed for the large scale identification of new driver mutations and potential targets for therapy. In 2004, pivotal studies showed that response to the tyrosine kinase inhibitor gefitinib was dependent on activating mutations in the EGFR gene [26,27]. Since then, the complex and highly heterogeneous genetic landscape of lung cancer has been

investigated through large scale sequencing studies [28,29]. In ADCs, many of the identified mutations are current or potential targets for therapy, such as EGFR, MET, ROS1, ALK, HER2 and BRAF. In contrast, alterations in FGFR1/2/3, DDR2 and PI3K have been identified as potentially targetable in SCC [30]. Interestingly, sequencing in SCC identified inactivating mutations in the HLA-A gene in a subset of patients, suggesting a direct role for somatic mutation in helping cancers avoid immune destruction [29].

1.1.5 Prevention

While smoking cessation and prevention remains the most important approach to reduce the mortality of lung cancer, screening is an attractive strategy. In 2014 the results of the US National Lung Screening Trial (NLST) were published, showing a 20 % reduction in lung cancer deaths and a 6% decrease in death of all causes with low-dose thoracic Computed Tomography (CT) [31]. Consequently, numerous US clinical guidelines now recommended yearly CT screening for individuals at high risk (≥ 30 pack-years, age 55 – 75 years) [32,33]. Several large European screening trials are due to be completed in the coming years, and most European countries including Norway are awaiting these result before deciding to implement a national lung cancer screening program [34,35].

Chemoprevention, which entails using dietary or pharmacologic interventions to prevent cancer, has been investigated in large randomized trials based on the epidemiological link between high vegetable consumption and reduced risk for lung cancer [36]. However, results have been disappointing and no form of chemoprevention is currently recommended for lung cancer [37].

1.1.6 Diagnosis and staging

Approximately one fourth of patients with lung cancer are asymptomatic at the time of diagnosis and are diagnosed incidentally. However, most patients display symptoms related to the primary tumor or local or distant metastasis and are likely to have more advanced disease [38].

The diagnostic work-up of lung cancer routinely includes chest x-ray, as well as a CT of the thorax and the upper abdomen [35]. If a suspected malignant tumor is detected a biopsy or cytological sample is needed for diagnosis. This can be obtained by either bronchoscopy, with CT-guidance, or by endobronchial or endoesophageal ultrasound. When indicated, mediastinoscopy, mediastinotomy, thoracoscopy or thoracentesis may be performed. A Positron emission tomography (PET) scan combined with CT is today recommended for most patients considered for surgical treatment or stereotactic radiotherapy to delineate the extent of mediastinal disease and possible distant metastasis. The performed diagnostic investigations determine the clinical stage, which in turn guides the choice of treatment. NSCLC is staged according to the TNM (Tumor, lymph Node, Metastasis) Classification of Malignant Tumours published by the Union for International Cancer

Control (UICC) [39]. The 7th UICC TNM edition was implemented in 2010, and is based on a retrospective database of 67,725 NSCLC patients collected by the International Association for the Study of Lung Cancer (IASLC) [40]. The 8th edition of the TNM classification was published in 2016 [41], and its enactment began January 2017. This new TNM classification is based on a new database of more than 77,156 lung cancer cases compiled by the IASLC [42]. While no changes have been made to the N-descriptors, new size cut points at 1 and 4 cm have been introduced for the T-category (Table 1). The introduction of new T-categories has led to the introduction of new stage groupings, with stage IA now further sub-classified to IA1, IA2, IA3 based on tumor size (when N0 and M0). A new stage (IIIC) has been introduced for the most advanced local disease categories (i.e. T3/T4, N3, and M0). Stage IV disease is now divided into IVA and IVB based on location and number of metastases [43].

Table 1: Stage groupings according to the eight edition of the TNM classification of lung cancer. Adapted from Goldstraw, P., et al., *The IASLC Lung Cancer Staging Project: Proposals for Revision of the TNM Stage Groupings in the Forthcoming (Eighth) Edition of the TNM Classification for Lung Cancer.* Journal of Thoracic Oncology, 2016 [43]

Stage	Sub-stage	T Category	N Category	M Category	5-year OS IASCL 2016[43]
Occult carcinoma		TX Primary tumor not assessed , or proven only by cells or imaging	N0 No regional lymph node metastasis	M0 No distant metastasis	
Stage 0		Tis Carcinoma *in situ*	N0		
Stage I	**IA1**	T1a(mi) Minimally invasive adenocarcinoma[a]	N0		90
		T1a Tumor ≤1 cm	N0		
	IA2	T1b Tumor >1 cm ≤2 cm	N0		85
	IA3	T1c Tumor >2 cm ≤3 cm	N0		80
	IB	T2a Tumor >3 cm ≤4 cm[b]	N0		73
Stage II	**IIA**	T2b Tumor >4 cm ≤5 cm[b]	N0		65
	IIB	T1a-c	N1[e]		56
		T2a	N1		
		T2b	N1		
		T3 Tumor >5 cm ≤7 cm[c]	N0		
Stage III	**IIIA**	T1a-c	N2[f]		41
		T2a-b	N2		
		T3	N1		
		T4 Tumor >7 cm[d]	N0		
		T4	N1		
	IIIB	T1a-c	N3[g]		24
		T2a-b	N3		
		T3	N2		
		T4	N2		
	IIIC	T3	N3		12
		T4	N3		
Stage IV	**IVA**	Any T	Any N	M1a[h]	
		Any T	Any N	M1b[i]	
	IVB	Any T	Any N	M1c[j]	

[a]: Solitary adenocarcinoma, ≤ 3cm with a predominately lepidic pattern and ≤ 5mm invasion in any one focus.
[b]: or tumor with any of the following features: Involves main bronchus regardless of distance from the carina but without involvement of the carina; invades visceral pleura; associated with atelectasis or obstructive pneumonitis that extends to the hilar region, involving part or all of the lung.
[c]: or associated with separate tumor nodule(s) in the same lobe as the primary tumor or directly invades any of the following structures: chest wall (including the parietal pleura and superior sulcus tumors), phrenic nerve, parietal pericardium.
[d]: or associated with separate tumor nodule(s) in a different ipsilateral lobe than that of the primary tumor or invades any of the following structures: diaphragm, mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, and carina.
[e]: Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension.
[f]: Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s).
[g]: Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node.
[h]: Separate tumor nodule(s) in a contralateral lobe; tumor with pleural or pericardial nodule(s) or malignant pleural or pericardial effusion.
[i]: Single extrathoracic metastasis.
[j]: Multiple extrathoracic metastases in one or more organs.

1.1.7 Treatment

Surgery is the main modality for the curative treatment of NSCLC. For selected patients, radiotherapy alone or in conjunction with chemotherapy or surgery can be curative. However, only approximately 30% of all NSCLC patients are candidates for curative treatment as most are diagnosed at an advanced stage or have co-morbidities that preclude definitive treatment [35].

1.1.7.1 Curative

Patients with stage I NSCLC are commonly treated with surgery alone, while radiotherapy may be used for those who are inoperable. For patients in stage II adjuvant chemotherapy is administered post surgery. The preferred adjuvant regimen in Norway is four cycles of cisplatin and vinorelbine [35]. Inoperable patients in stage II can receive radiotherapy, which can be supplemented with chemotherapy. Radiotherapy is increasingly given stereotactically if feasible (tumor size <6 cm), as treatment time and toxicity is reduced compared to conventional fractionation [35]. For operable stage I patients, comparable results have been reported for stereotactic radiotherapy compared to surgery, though more evidence is needed to confirm these findings [44,45]. For operable stage III patients with N0 or N1 disease the recommended treatment is surgery with adjuvant chemotherapy, post-operative radiotherapy is recommended in current Norwegian guidlines when the surgical margins are not free or if N2 disease is detected during surgery. Inoperable stage III patients are considered for potentially curative radiotherapy, which may be given concomitantly with chemotherapy. For stage III patients with negative prognostic factors (e.g. ECOG 2 ≥, weight loss, high age) treatment with curative treatment may not be feasible and only palliative treatment is offered [35]. Neoadjuvant chemotherapy and radiotherapy is not recommended in the standard treatment of NSCLC, however it is an option for the potentially curative treatment of tumors in the superior sulcus (i.e. Pancoast-tumor) [46].

1.1.6.2 Advanced disease

Most NSCLC patients present with advanced disease and are not candidates for curative treatment. For advanced disease, chemotherapy has been the mainstay of treatment, commonly given over 3-4 courses in the form of doublet of a platinum agent and vinorelbine, gemcitabine, pemetrexed (non SCC only) or docetaxel. The recommended doublet in Norwegian guidelines is carboplatin and vinorelibine, based on favorable toxicity profile and cost-effectiveness. For patients receiving a doublet containing pemetrexed, maintenance therapy has been shown to improve overall survival and is currently recommended in Norwegian guidelines [35]. While the angiogenesis inhibitor bevacizumab is recommended in addition to chemotherapy in patients with non-SCC in US and European treatment guidelines [24], current Norwegian guidelines recommend against its use based on the limited effects seen on survival in the AVAIL-trail [47]. On average, chemotherapy in advanced

NSCLC translates to a 1-year survival gain of 9% and a 1.5 month increase in median survival, in addition to improved quality of life [48].

Approximately 10% of Norwegian NSCLC patients with ADC are EGFR positive and are recommended a Tyrosine kinase inhibitor (TKI) as first line of therapy [35]. For patients harboring the ALK translocation, the TKI crizitonib is recommended in the first line [14]. Despite impressive response rates, resistance invariably develops in patients receiving targeted therapy and the majority relapse within 12 months [14,49]. For EGFR+ patients with the T790 resistance mutation (49%–60% of patients), the third generation TKI osimertinib is recommended as second line treatment [14]. Similarly, the potent second generation ALK-inhibitor alectinib is recommended after progression for patients previously treated with crizitonib [14].

Recently, immunotherapy has shown efficacy in advanced NSCLC in form of inhibitors of the immune checkpoints Programmed cell death protein 1 (PD-1) and Programmed death-ligand 1 (PD-L1). The PD1 inhibitors nivolumab and pembrolizumab are currently recommended for the second line treatment of advanced NSCLC, based on improved overall survival, response rate and tolerability compared to single agent docetaxel [14]. Notably, some patients exhibit durable responses and long term remissions [50]. The use of assays to quantify PD-L1 expression on tumor tissue can enable the selection of patients who are more likely to respond, and a positive assay result is a requirement for treatment with pembrolizumab. However, these assays are controversial as some patients with negative assay results may respond to treatment, and their utility has not been uniform across all clinical trials [50]. Both nivolumab and pembrolizumab are currently approved for advanced NSCLC in Norway [51]. Recently, pembrolizumab has also been approved for the first line treatment of NSCLC by the FDA, based on a landmark trial were PDL1+ positive patients treated with pembrolizumab had longer progression-free and overall survival compared to those treated with conventional platinum-based chemotherapy [52]. A multitude of further studies are being conducted on PD1/PDL1 as inhibitors as single agents as well in combination with other immunotherapies (e.g. CTLA-4 inhibitors and LAG-3 inhibitors), chemotherapies or radiotherapy for the treatment NSCLC [50].

In the palliative setting, radiotherapy is an important treatment option for reduction of symptoms related to thoracic disease, which is also used in the palliative treatment of bone and brain metastasis. For patients with central airway obstruction, endoscopic interventions are increasingly utilized as they provide more rapid symptom relief than conventional radiotherapy and chemotherapy [35].

1.2 Tumor stroma

While activating mutations in cancer cells are the driving force of cancer development, different cells and molecules in the tumor's microenvironment (tumor stroma) are increasingly recognized as crucial contributors to tumor growth, development and metastasis [53]. The tumor stroma consists of non-malignant cells such as fibroblasts, adaptive and innate immune cells, blood vessels with endothelial cells and pericytes, and the extracellular matrix consisting of proteins and proteoglycans [54] (Figure 3). Cancer cells can produce growth factors that modulate normal stromal cells and induce a cancer-promoting microenvironment. Fibroblasts in the tumor stroma (cancer associated fibroblasts, CAFs) can stimulate tumor growth, invasion, angiogenesis and metastasis [30]. The formation of new blood and lymphatic vessels allows for influx of innate and adaptive immune cells. As discussed below, the immune cells in the tumor microenvironment can both stimulate and hamper cancer development through multiple mechanisms and mediators.

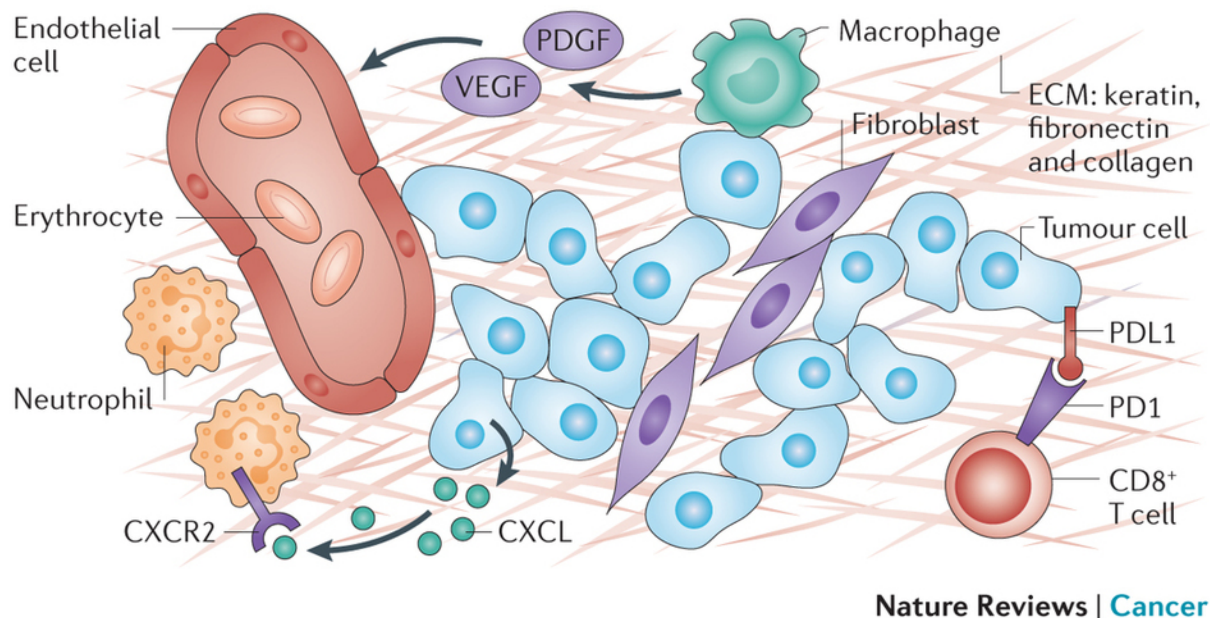

Figure 3. The lung cancer microenvironment. The lung cancer microenvironment has an important role in determining characteristic of a malignant lung tumor. Blood vessels can by formed by recruitment of endothelial via factors such as PDGF and VEGF. As vessels are formed, numerous immune cells infiltrate the tumor microenvironment. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer, [30], copyright 2014.

1.3 The immune system and cancer

The immune system plays a dual role in the development and progression of cancer [55]. Immune cells may aid and stimulate cancer growth, but also inhibit cancer progression by initiating and sustaining an anti-tumor response. In the 2010 update to the seminal paper "Hallmarks of Cancer", Hanahan and Weinberg recognized evading immune destruction as an emerging hallmark of cancer, while tumor-promoting inflammation was recognized as an enabling characteristic of cancer development [56]. Although the immune system can and sometimes does inhibit cancer formation, it is obvious that the immune response often is not sufficient to halt cancer growth and spread. Reactivating or boosting the intrinsic tumor-suppressing capabilities of the immune system is the basis of various current and prospective treatments of cancer [57].

1.3.1 Inflammation

A putative connection between cancer and inflammation has been recognized since Rudolf Virchow in 1863 observed that cancer often develops in tissues exposed to chronic inflammation [58]. It is now accepted that innate immune cells can play a significant part in cancer development through tumor promoting inflammation [56]. Inflammation releases bioactive molecules to the tumor microenvironment. These include growth factors which stimulate proliferative signaling, survival factors inhibiting cell death, angiogenic factors as well as enzymes that can modify the extracellular matrix to promote invasion and metastasis [56]. Additionally, chronic inflammation perpetuated by innate immune cells may contribute to a mutagenic microenvironment conductive to malignant transformation [59]. Tobacco smoke contains various carcinogens that directly promote cancer development. However, tobacco smoke has also been shown to indirectly promote lung cancer development through induction of inflammation in mice [60]. Chronic obstructive pulmonary disease (COPD), which is associated with chronic inflammation of the airways, is an independent risk factor for lung cancer [61]. In experimental models, lung cancer cells can promote tumor progression and metastasis by activating macrophages that generate an inflammatory microenvironment [62,63].

1.3.2 Immunoediting

The dual role of the immune system in cancer is encapsulated in the concept of immunoediting; a continual process where the immune system both protects against and stimulates tumor development. As delineated by Schreiber and colleagues, immunoediting involves three phases: elimination, equilibrium and escape [64]. In the elimination phase cells and mediators from innate and adaptive immune cells work in concert to eliminate a tumor before it becomes clinically evident. This phase is analogous to the older concept of immunosurveillance [64]. If the immune system does not

successfully eradicate the tumor an equilibrium may develop, where the immune system keeps the tumor in check and prevents net outgrowth. In this phase the tumor can become functionally dormant and remain clinically undetected [65] . Finally, the tumor cells may escape dormancy and develop into clinically manifest disease. Various mechanisms may contribute to tumor escape including reduced immune recognition, increased resistance or survival (through genetic or epigenetic changes), or the development of an immunosuppressive microenvironment [66-68].

1.3.3 The anti-tumor immune response

While innate immune cells can support cancer formation through tumor-promoting inflammation, they can also contribute to tumor control directly or indirectly by activating adaptive immunity [69] . Direct innate mediators include NK-cells and NK-T-cells, while dendritic cells are important to the activation of T-cells, who in turn are central effectors of the adaptive anti-tumor immune response [69]. The prototypical T-cell mediated immune response begins at the tumor site [70]. Here, antigen-presenting cells (APCs, e.g. dendritic cells) take up tumor associated antigens (TAA) and process them. To promote immunity rather than tolerance a maturation signal is needed for APCs, such as pro-inflammatory cytokines or factors released form necrotic tumor cells [71]. APCs then migrate to tumor draining lymph in order to prime T-cells, though evidence suggests this might occur also locally in the tumor stroma in tertiary lymphoid structures (TLS) [72]. APCs then present processed antigens on MHC molecules to T-cells, which may result in priming and activation of an effector T-cell response. The effector T-cells may then traffic back to the tumor to specifically recognize and kill cancer cells [70]. However, multiple mechanisms exist for tumors to inhibit and ultimately avoid immune destruction [70].

1.3.4 Immune suppression and tumor escape

Reduced immune recognition may be the result of the ongoing immune response against a tumor. Evidence from studies in mice show that T-cells that recognize TAA on cancer cells may contribute to the selection and expansion of antigen negative cancer cells who are not recognized by the immune system, leading to tumor escape [67,68]. Additionally, components of the antigen processing machinery (e.g TAP 1/2 and MHC molecules) is frequently downregulated in lung cancer, inhibiting the recognition of tumor cells by T-cells [73]. While this down-regulation frequently occurs through epigenetic mechanisms, it can also occur through mutation[73].

Epidemiological evidence supports a role for the immune system in inhibiting lung cancer tumorigenesis. After adjustment for smoking, the risk of lung cancer is increased 2 to 4-fold for HIV+ positive patients [74]. In a meta-analysis, patients who were immune suppressed because of organ transplantation and patients with HIV/AIDS had an increased risk of NSCLC [75].

Cancer cells can secrete factors that inhibit adaptive and innate immune cells, and create an immunosuppressive microenvironment conductive to tumor escape [64]. Release of cytokines such as GM-CSF, IK-1B, VEGF and PGE2 stimulate expansion and proliferation of myeloid-derived suppressor cells (MDSCs). In NSCLC, MDSCs are implicated in suppression of CD8+ effector T cells, and increasing levels of MDSCs in patient plasma has been associated with reduced response to chemotherapy [76].

Regulatory T-cells (Tregs) inhibit effector T-cell functions and are important for maintenance of tolerance against self-antigens, limiting immune responses and preventing auto-immunity [64]. In cancer, Tregs are implicated in suppression of anti-tumor immune responses. The induction of Tregs can be supported by cytokines (e.g TGF-β) in the tumor microenvironment produced by tumor cells [77]. In murine models of NSCLC, Tregs can inhibit anti-tumor T-cell responses [78] and support cancer development [79].

The expression and up-regulation of various immune checkpoints can contribute to suppression of T-cell function [80]. T-cell maturation and activity is regulated by interactions between different co-stimulatory and co-inhibiting receptors and ligands [81]. The negative immune regulators (immune checkpoints) are essential for self-tolerance under normal physiological conditions and limit tissue damage during inflammation [80]. Cytotoxic T lymphocyte antigen 4 (CTLA-4) is the prototypical immune checkpoint receptor and serves to regulate the amplitude of T-cell activation and mediating the immune suppressive function of Tregs [81]. In addition to CTLA-4, several other immune checkpoints have been characterized such as programmed cell death protein 1 (PD-1), lymphocyte activation gene 3 (LAG-3), T cell immunoglulin domain and mucin domain 3 (TIM-3) and T cell immunoglobulin and ITIM domain (TIGIT) [82]. Seminal studies in mice showed that inhibiting CTLA-4 by blocking antibodies could induce antitumor immunity and tumor regression [83,84]. This has led to the clinical development and approval of CTLA-4 inhibitors for the treatment of cancer [85]. Similarly, inhibition of the immune checkpoint PD1 and its ligand PDL-1 has shown efficacy in multiple solid cancers, including NSCLC [86,87].

1.3.6 Chemokines

Chemokines are chemotactic cytokines who are central in regulating the migration and positioning of leucocytes under both physiological and pathological conditions [88]. The differential expression of chemokine receptors on leucocytes allow for recruitment of specific cell types under particular physiological conditions. Chemokines are important regulators of the development of T-cells in the thymus, and for the normal development of B-cells, monocytes, macrophages, neutrophils and NK cells in the bone marrow [88]. In addition to their role in immunity, chemokines are involved in embryonic development, wound healing, angiogenesis and cancer [89].

Chemokines play a complex and multifaceted role in cancer development. By recruiting immune cells to the tumor microenvironment, chemokines can contribute to an anti-tumor immune response [90]. For example, the chemokine CXCR3 is central for recruitment of NK-cells and effector T-cells to tumors [91,92]. Other chemokines, such as CCL19, CCL21 and CXCL13 can contribute to formation of TLS, which are associated with improved survival in NSCLC [72]. However, chemokines can also stimulate tumor progression, angiogenesis and metastasis [93]. Cancer cells and stromal cells can secrete chemokines (e.g. CCL2 and CCL5) that attract MDSCS who in turn promote a tumor suppressive microenvironment [94]. In breast cancer, the chemokine CCL2 can attract inflammatory monocytes expressing the receptor CCR2 who further develop into macrophages that promote metastasis to the lung or bone [95]. Various chemokines have been suggested to play a part in the development and progression of NSCLC [96]. In particular, the chemokine CXCR4 and its receptor CXCL12 have been shown to stimulate invasiveness and metastatic potential of NSCLC cells, and the over-expression of CXCR4 in tumor specimens is to linked poor survival [97,98].

1.3.7 Infiltrating immune cells in NSCLC

Infiltration of leucocytes from both the innate and adaptive immune system is a common finding in most cancers, including NSCLC [99]. A diverse range of immune cells may be found in cancer, including macrophages, mast cells, dendritic cells, NK-cells, B-cells and T-cells. These immune cells can in turn be divided into subsets based on the differential expression of various surface markers and receptors. Among T-cells, subsets include T-helper cells (e.g. T_{H1}, T_{H2}, T_{H17}), Tregs, T follicular helper cells and cytotoxic T-cells (CD8+ T cells). Infiltrating immune cells may be located in the tumor core, the invasive margin, in TLS or in the tumor stroma [100]. Together, the type, density and location of immune cells within a tumor define the "*immune contexture"* [101]. This immune contexture is shaped by a complex interplay between tumor cells, immune cells, stromal cells and chemokines and cytokines in the tumor microenvironment. For many human cancers, there exists a strong association between components of the immune contexture and clinical outcome [102]. In particular, a strong infiltrate of TILs has been associated with an improved clinical outcome in cancers such as melanoma, colon, head and neck, breast, bladder, urothelial, ovarian and lung cancer [100].

Infiltrating immune cells in NSCLC include T-cells, B-cells, NK-cells, dendritic cells, macrophages, neutrophils and macrophages. As previously mentioned, immune cells in NSCLC can be organized in TLS. These are composed of a B-cell follicle with follicular helper T-cells, macrophages, follicular dendritic cells and a T-cell area with mature dendritic cells. In NSCLC, a positive correlation has been observed between high densities of TLS, mature dendritic cells and improved survival [103]. In a recent meta-analysis of TILs in NSCLC, high levels of CD8+ T-cells in stromal and epithelial compartments were associated with improved overall survival [104]. In contrast, FOXP3+ T cells (Tregs) were

associated with a worse outcome. However, significant heterogeneity existed between studies concerning cutoffs, location of the immune infiltrate (i.e. invasive margin or central tumor), sample size and follow-up.

1.3.8 Immunoscore – supplementing the TNM

The TNM classification guides clinical decision making and is the predominant prognostic factor in NSCLC [105]. The tumor profile, i.e. the histopathological-, molecular-, hormonal- and genetic characteristics of a tumor, can supplement the TNM classification. In NSCLC, some factors (e.g. EGFR mutation, PD-L1 expression, histology) predicts response to therapy, whereas patient-related factors such as age, sex and performance status informs about prognosis and may guide treatment [35]. Nevertheless, patient outcomes can vary significantly within each stage, highlighting the need for new prognostic factors to complement the TNM staging.

In colorectal cancer, as in NSCLC, numerous studies have shown that quantifying the *in situ* immune infiltrate in resection specimens predict patient outcome and supplement the TNM [106,107]. Galon and colleagues have led efforts to translate these findings into the clinic by means of a standardized immune cell score, termed immunoscore. The immunoscore is based on quantification of CD3+ and CD8+ T-cells in two compartments of resection specimens (central tumor and invasive margin) by an image analysis workstation [108]. The colon cancer immunoscore was recently validated in a retrospective study led by an international consortium, and showed significantly longer time to relapse for patients with a high Immunoscore in both the training set and in independent validation sets compared to patients with a low score [109]. It has been suggested that the implementation of the Immunoscore can lead to a new classification of cancer, termed TNM-I (TNM-Immune) [108]. However, it is worth noting that that the Immunoscore cannot be incorporated directly into the TNM, as the TNM by definition is an anatomic description of the extent of disease. Consequently, no molecular factor or marker may improve it [110]. Nevertheless, quantifying immune markers in an immunoscore is an attractive strategy for supplementing the TNM, and may allow for improved prognostic stratification and support for treatment decisions in NSCLC[111].

1.4 Immune-related markers in different patient groups

There exists a multitude of immunological markers of potential biological and clinical relevance in NSCLC; however, it is beyond the scope of this thesis to cover them all in depth. Below, aspects of selected immunological markers and patients groups of particular relevance to this thesis are presented.

1.4.1 The *in situ* immune infiltrate in patients treated with adjuvant radiotherapy.

The rationale for adjuvant postoperative radiotherapy (PORT) in NSCLC is reduced local recurrence and improved survival. However, a recent Cochrane review showed an adverse effect on survival by PORT in completely resected NSCLC [112], though many of the included trials used technology and dosages not relevant to current clinical practice [113] . Retrospective data from the US National Cancer Database supports the use of PORT in NSCLC patients with N2 disease [113], as is also recommended in current Norwegian guidelines [35]. Evidence also supports the use of PORT in patients with positive margins after surgical resection [114]. Radiotherapy exerts its main effects by directly damaging tumor cells. However, evidence suggests that radiotherapy also contributes to systemic antitumor immunity [115]. Additionally, radiotherapy and immunotherapy can have synergistic effects in cancer treatment [115]. Different adaptive and innate immune markers have been shown to have prognostic impact in NSCLC [116,117], however their impact on survival in patients treated with radiotherapy remains unclear.

1.4.2 The chemokines CXCL16 and its receptor CXCR6

CXCL16 is a transmembrane chemokine and the only known ligand in for the receptor CXCR6 [118]. While soluble CXCL16 can stimulate the recruitment and adhesion of cells expressing CXCR6 [119], it can also act as a scavenger receptor for oxidized low-density lipoprotein [120]. In addition to their role in leukocyte recruitment, evidence suggests that CXCL16 and CXCR6 play diverse roles in cancer. These markers have been linked to tumor promoting inflammation [121] and angiogenesis [122], but also to recruitment of leucocytes to tumors [123]. Accordingly, disparate results exist for the impact of CXCL16 and CXCR6 on prognosis, with their expression linked to both reduced [121] and improved [124] survival in different cancers.

1.4.3 Stromal CD8

CD8+ T cells play a central role in clearing viral, protozoan and bacterial pathogens [125]. They also have an essential role in antitumor immunity [71]. Effector CD8+ T-cells can hamper cancer development through direct killing of cancer cells and by releasing cytokines (e.g. IFN-γ) that inhibit tumor growth

[126]. It has also been suggested that CD8+ T-cells can contribute tumor regression by exerting their cytotoxicity in the tumor stroma [126] . The importance of CD8+ T cells in cancer is reflected by the multiple existing and proposed therapeutic strategies for increasing the level and quality of CD8+ T-cell responses [70,127]. Additionally, with an exception for renal cancer, increasing levels of CD8+ T-cells in resection specimens are linked to improved prognosis in the majority of human cancers [102]. We have previously analyzed expression of CD8 in 335 NSCLC specimens, and found that increased levels of CD8+ cells in the stromal compartment of tumors were independently associated with improved survival [116].

1.4.4 LAG-3

The lymphocyte activation gene (LAG-3), originally identified in T-cells, can also be expressed by NK cells, B-cells and plasmacytoid dendritic cells. As an immune checkpoint molecule, LAG-3 has a role in the negative regulation of T-cell expansion and function [82]. Studies in mice suggest LAG-3 acts synergistically with PD-1 to prevent autoimmunity [128]. Dual expression of PD-1 and LAG-3 on TILs has been noted in pre-clinical cancer models, and dual inhibition of PD-1 and LAG-3 can lead to improved anti-tumor T-cell responses [82]. Stage I trials utilizing LAG-3 blocking antibodies for the treatment of cancer are currently underway in NSCLC and other solid malignancies [129]. Few large-scale studies have examined the prognostic impact of LAG-3 in resection specimens. In NSCLC, LAG-3 has been correlated with a worse prognosis in univariate survival analysis in one study of 139 resected patients; however, this finding did not remain significant in multivariate analysis [130].

1.5 Tissue microarray

Tissue microarrays (TMAs) enable the simultaneous investigation of a large number of tissue specimens on a single histological slide. Battifora introduced the concept of a multitumor tissue block (the “sausage block”) in 1986, further refining the method in 1990 (the “checkerboard tissue block”) [131,132]. In 1998, Kononen et al. published the first modern TMA study, presenting a rapid and reproducible method for producing quality TMAs [133]. Since then, TMA technology has seen widespread adoption and has become a standard instrument in tissue based research [134]. TMAs are commonly constructed from minute tissue cores from formalin fixed and paraffin-embedded tumor specimens (donor blocks). The tissue cores are then transferred to a recipient block according to a fixed matrix, allowing for reliable allocation of clinical and pathological data to the individual tissue “cores” on the histological slide [134]. TMAs may also be constructed from other sources such as frozen tissues, cell lines or needle biopsies [135]. Methods such as immunohistochemistry and various forms of *in situ* hybridization are utilized on TMAs to investigate biomarkers at the DNA, RNA or protein level. Depending on the utilized tissue and associated clinical data, applications of TMAs in cancer research include the investigation of prevalence and cellular localization of molecular alterations and the investigation of biomarkers and their relation to tumor stages, patient prognosis or response to therapy [134].

1.6 Immunohistochemistry

Immunohistochemistry (IHC) is an important tool for the detection of specific antigens on tissue sections which is widely adopted in medical research and diagnostics [136]. Invented in the 1940s, today there are numerous protocols for IHC applications and assays [137]. The general IHC assay involves using a primary antibody capable of specifically binding epitopes of a given antigen. A secondary reporter-coupled antibody specific for the primary antibody is then added. Finally, the addition of a substrate that reacts with the reporter molecule results in a colored precipitate, allowing for visualization of the antigen-antibody-complex. Alternatively, the primary antibody may be conjugated to a reporter molecule directly, though this may cause reduced sensitivity [136]. An advantage when using IHC on TMAs is that the various parts of the experiment such as antigen retrieval, temperature, incubation times, washing procedure, and reagent concentration are standardized and not subject to inter-batch variability.

1.6.1 Antibodies

Selection of an appropriate antibody is an important step when conducting any IHC based study [138]. Primary antibodies are of two major types: polyclonal or monoclonal. Polyclonal antibodies are generated by immunizing animals (e.g. rabbits or goats) resulting in a mixture of antibodies capable of binding different epitopes of the chosen antigen. This in turn leads to higher sensitivity for detection a given antigen, though the risk of cross-reactivity may increase. Monoclonal antibodies are generated from hybridomas made by immortalizing a single B-cell clone by fusing it to a myeloma cell line. As they are made from a single B-cell clone, monoclonal antibodies recognize a single epitope of the antigen. Accordingly, monoclonal antibodies have a reduced risk of cross-reactivity. However, the binding affinity of a monoclonal antibody is dependent on the conformation of a single epitope, which may be altered by experimental factors such as temperature, pH and fixation [139]. Consequently, monoclonal are more likely to work only in optimal experimental conditions, and the risk of false negative assay results are higher. Their monospecificity can also be an important advantage, making it easier to examine changes in phosphorylation states, molecular conformation and protein-protein interactions [139]. Another advantage of monoclonal antibodies is that once a hybridoma has been constructed, antibodies can be continuously generated with high lot-to-lot consistency. Polyclonal antibodies generated from different animals may differ with regards to which epitopes they recognize and their avidity may change over time.

2. AIMS OF THE THESIS

The aims of this thesis were to explore the expression of selected immune-related markers in different groups of NSCLC patients on TMAs, thereby illuminating their role and prognostic impact in NSCLC. Specifically, we aimed to:

- Explore the prognostic impact of adaptive and innate immune markers in patients treated with postoperative radiotherapy.
- Investigate the prognostic impact of the chemokines CXCR6 and CXCL16, and their relation to other immune markers.
- Validate stromal CD8 as a prognostic marker in resected NSCLC.
- Explore the prognostic impact of LAG-3 in primary tumors and metastatic lymph nodes, and its potential for inclusion in an NSCLC immunoscore.

3. MATERIALS AND METHODS

3.1 Patient cohorts

Our original cohort (paper 2 and 3) consist of consecutive NSCLC patients who underwent radical resection for clinical stage I-IIIA NSCLC at the University Hospital of North Norway (UNN), Tromsø, Norway and Nordland Hospital (NH), Bodø, Norway from 1990 through 2005 (n = 335). In paper 1, only patients who received postoperative radiotherapy (n = 55) were included for analysis. The original cohort was expanded in 2013 to include patients resected from 2005 through 2011 (n = 219). The patient material was simultaneously expanded to include lymph nodes for the 172 patients with lymph node metastasis; adequate paraffin-embedded tissue specimens were available for 143 of the patients. For all patients, formalin-fixed and paraffin-embedded tissue blocks were obtained from the pathological departments of the respective hospitals. Clinical patient data were registered from hospital databases.

Papers 1, 2 and 3 include follow up data as of January 2011. For paper 4, follow up data as of Oct. 1 2013 is included. For papers 1, 2 and 3 the pathological staging was done according to the 7th edition of the UICC TNM classification, the tumors collected before 2010 were reviewed and restaged from the previous 6th UICC TNM classification. For the papers 1, 2 and 3, histological grading and subtyping was done according to the 2004 World Health Organization guidelines (WHO) [140]. For paper 3, bronchialveolar carcinomas (BAC) ≤ 3 cm were reclassified as adenocarcinoma in situ (AIS), without new assessment of the tumors, on the basis of the 2011 IASLC classification of lung adenocarcinoma [141]. For paper 4, staging for all patients was updated to conform to the recently implemented 8th UICC TNM classification, resulting in 21 patients being staged as IIIB. Additionally, two pathologist re-reviewed all histological slides and performed subtyping according to the new 2015 WHO guidelines, utilizing immunohistochemistry when appropriate.

A total of 633 stage I-IIIA[*] NSCLC patients resected between 1990 to 2011 were potentially eligible for inclusion in our cohort (paper 4). Exclusion criteria were preoperative chemotherapy or radiotherapy (n = 15), other malignancy within 5 years prior to NSCLC diagnosis (n = 39) or inadequate paraffin-embedded surgical specimens (n = 26). In paper 3, patients with BAC ≤ 3 cm were also excluded (n = 11), in papers 1-2 the BACs were included in the adenocarcinomas. However, following the histological re-review to conform to the 2015 WHO guidelines it was revealed that the 11 previously excluded BACs ≤ 3 cm in our cohort, presumed to represent adenocarcinoma *in situ*,

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[*] According to the 7th UICC TNM classification.

were in fact invasive carcinomas. Consequently, these patients were re-included in the cohort for paper 4.

Our current database (as of Dec 31, 2016) consists of 553 patients (335 from original cohort, 218 from the expansion). In paper 3, two additional cohorts of stage I-IIIA NSCLC were included for validation: these were from Oslo University Hospital, the Norwegian Radium Hospital (OUS), Oslo, Norway (*n* = 295), and Odense University Hospital (OUH), Odense, Denmark (*n* = 178).

3.2 TMA construction

All included samples were subject to careful histological review by an experienced pathologist. The most representative paraffin blocks were selected, and two areas of neoplastic epithelial cells and two from tumor stroma were marked on hematoxylin and eosin (H&E) slides to guide sampling for the recipient TMA blocks. A tissue-arraying instrument (MTA-1, Beecher Instruments, Silver Springs, USA) was utilized to construct the TMAs. To perform the assembly, the recipient block was placed in a holder. Using the marked H&E slide as a guide, the donor block was punched to a retrieve 0.6 mm cylindrical tissue core, before a stylet guide the extrusion of the tissue core into the recipient block. From each primary tumor two cores of neoplastic epithelium and two of tumor stroma were included. Two cores were included from one suitable metastatic lymph from the patients with lymph node metastasis. In addition, normal tissue distant from the primary tumor was included in the TMAs to serve as controls for tissue staining. Fifteen TMA blocks were constructed, twelve of primary tumor and three of metastatic lymph nodes. From these blocks, 4-µm sections were cut with Micron microtome (HM355s), with individual slides containing up to 288 separate tissue cores. The TMA construction in the Danish [142] and Oslo [143] cohorts was mostly similar, however a core size of 1 mm was used. For the Oslo cohort the number of cores per patient included was variable (minimum three cores), in the Danish cohort two cores from the central tumor and two from the invasive margin were included.

3.3 IHC procedure

For paper 1, antigen retrieval was performed by placing the specimens in 0.01 m citrate buffer at pH 6.0 and exposing them to repeated (2) microwave heating of 10 min at 450 W. The slides were incubated with antibodies in the Ventatna Benchmark, XT automated slide stainer. Endogenous peroxidase was blocked using the Dako EnVision+ System-Horseradish Peroxidase [diaminobenzidine (DAB)] kit (Dako, Glostrup, Denmark). The DAB kit was used to visualize the antigens by application of liquid diaminobenzidine and substrate-chromogen, yielding a brown reaction product at the reaction site.

For paper 2, Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxidase for 10 minutes. Sections were blocked in 5% goat or rabbit serum for 1 hour before overnight incubation with the primary antibodies at 4ºC. The primary antibodies were visualized by adding a secondary biotin-conjugated antibody followed by an Avidin/Biodin/Peroxidase complex (Vectastain ABC Elite-kit, Vectastain) and substrate (Vector NovaRed, Vectastain).

For paper 3, endogenous peroxidase activity was quenched using 3% hydrogen peroxide in all cohorts. In the Norwegian cohorts, the Ventana Benchmark, XT automated slide stainer was used for

immunohistochemistry (IHC). The slides were baked for 60°C overnight, deparaffinized, and submitted to heat-induced epitope retrieval. Cell conditioning-1 protocol (CC1 Mild) for 30 minutes at 95°C epitope retrieval was used for Tromsø/Bodø cohorts and CC1 Standard for 60 minutes at 95°C for the Oslo cohort. After application of primary antibody the slides were incubated for 32 minutes at 36°C, followed by washing in buffer and visualization. Visualization was done with the Iview DAB Detection Kit for Tromsø/Bodø cohorts and the ultraview DAB Detection Kit for the Oslo cohort. In the Danish cohort, the Ventana Benchmark, Ultra automated slide stainer was utilized. Slides were baked at 75°C for 4 minutes, deparaffinized in EZ Prep, and submitted to heat-induced epitope retrieval. CC1 Mild for 36 minutes at pH 8.5 and 99°C was used. The primary antibody was applied and the slides were incubated for 32 minutes at 36°C, followed by washing in buffer and visualization using the OptiView DAB Detection Kit. For all cohorts 3,3'-diaminobenzidine was used as the chromogen. To validate the different IHC procedures, additional TMA slides from the Danish cohort were stained with the procedure used for the Oslo cohort.

For paper 4, staining was performed with the Ventana Discovery-ULTRA autostainer. Antigen retrieval was done with Ventana Ultra Cell conditioning for 40 minutes a t95°C. Sections were incubated antibody for 44 min at 36°C. The secondary antibody (UltraMap anti-rabbit HRP, #760-4315, Ventana) was loaded for 20 minutes, followed by 8 minutes of HQ-HRP amplification. Visualization was done with the discovery purple kit (#760-229, Ventana) with 32 min incubation. Counterstaining was performed using hematoxylin II (#790-2208, Ventana) for 28 minutes and then with a bluing reagent (#760-2037, Ventana) for 4 minutes.

3.3.1 Antibodies

The antibodies used in paper 1 were subject to validation by the manufacturer for IHC analysis on paraffin-embedded sections. In paper 2, we validated the antibodies by western blots combined with siRNA knockdown. For paper 3, well validated CD8 antibodies that are in routine clinical use were utilized. For paper 4, validation was performed with western blots of overexpressed cell lysate. For all antibodies, we chose positive tissue controls according to the manufacturers' recommendation. Negative reagent control was done by replacing the primary antibody with an antibody diluent. Only antibodies validated and recommended by the manufacturer for IHC analysis on paraffin-embedded material were used for the papers presented in this thesis (Table 1).

Table 1. Antibodies

Antibody	Vendor	Clone	Host species and clonality	Primary antibody titer
Paper 1				
CD1a	Ventana (Roche)	JMP	Mouse monoclonal	Prediluted
CD3	Ventana (Roche)	PS1	Mouse monoclonal	Prediluted
CD4	Novacastra	1F6	Mouse monoclonal	1:5
CD8	Ventana (Roche)	1A5	Mouse monoclonal	Prediluted
CD20	Ventana (Roche)	L26	Mouse monoclonal	Prediluted
CD56	Ventana (Roche)	123C3.D5	Mouse monoclonal	Prediluted
CD68	Ventana (Roche)	KP1	Mouse monoclonal	Prediluted
CD117	Ventana (Roche)	9.7	Mouse monoclonal	Prediluted
CD138	Ventana (Roche)	B-A38	Mouse monoclonal	Prediluted
M-CSF	Santa Cruz biotechnology	polyclonal	Rabbit polyclonal	1:25
CSF-1R	Santa Cruz biotechnology	polyclonal	Rabbit polyclonal	1:5
Paper 2				
CXCL16	Abcam	polyclonal	Rabbit polyclonal	1:100
CXCR6	Abcam	polyclonal	Goat polyclonal	1:100
Paper 3				
CD8[a]	Ventana (Roche)	1A5	Mouse monoclonal	Prediluted
CD8[b]	Roche	SP57	Rabbit monoclonal	Prediluted
CD8[c]	Dako	C8/144B		Prediluted
Paper 4				
LAG-3	Cell Signaling	D2G4O	Rabbit monoclonal	1:50

[a] Used in the UNN (Tromsø) and NH (Bodø) cohorts.
[b] Used in the OUS (Oslo) cohort and for the second staining of the OUH (Odense, Denmark) cohort.
[c] Used for the first staining of the OUH (Odense, Denmark) cohort.

3.4 Scoring

In the present studies, the biomarkers examined have been scored on a four-tiered ordinal scale, representing the intensity or density of staining. For density arbitrary cutoffs of 1%, 5%, 25% or 50% for each cell/compartment were chosen as these percentages are simple to follow. For intensity, were applicable, the following scale of staining was used 0=absent, 1=weak, 2=intermediate, 3=strong. Depending on the marker and its pattern of expression, staining was assessed in the tumor epithelium (cancer cells), the tumor stroma adjacent to tumor epithelium, or in the immune cells localized to the intraepithelial compartment (enclosed by tumor epithelial cells).

For all papers, the stained sections were scored independently by two scorers. For papers 1 and 2, the scorers were experienced pathologists. For paper 4, scoring was performed by two experienced microbiologists under supervision of an experienced pathologist. For paper 3 scoring was performed by two experienced pathologists in the Norwegian cohorts, and one pathologist and one oncologist in the Danish cohort. Additionally, as previously mentioned, the Danish cohort was reevaluated and scored by the same pathologist as in the Norwegian Oslo cohort for validation of the scoring procedure.

In paper 1, most of the examined immune cell markers showed homogenous staining intensity and were accordingly scored according to density (percentage of positive cells compared to the total amount of nucleated cells) in the tumor epithelial (T) and stromal (S) tissue compartments and dichotomized to low and high expression. For the following markers, the percentage of stained cells in the respective compartments determining high expression are given in parenthesis: CD1a (S ≥1%, T ≥1%), CD3 (S >50%, T ≥1%), CD4 (S ≥25%, S ≥5%), CD8 (S >50%, T >5%), CD20 (S ≥1%, T ≥1%), CD68 (S ≥25%, T ≥1%), CD138 (S >25%, T >5%). CD56 expression was rare in both tumor and stromal compartment and was scored only as present (high) or absent (low). Similarly, CD117 was only scored as present or absent, but only in the stromal compartment. CD138 was also expressed in the tumor cells were it was scored according to intensity, high expression was defined as a score >1. M-CSF and CSF-1R were scored according to intensity in the tumor cells, with high expression defined as a score ≥1.5 for both M-CSF and CSF-1R. In the stromal compartment, the intensity score was added to density score before dichotomization. The cell density score of the stroma was defined as the ratio of positive cells compared to the surface area of the extracellular matrix: 1 = low density (<25% cell/matrix ratio); 2 = intermediate density (25–50%) and 3 = high density (>50%). High expression was >3.5 for M-CSF and >3 for CSF-1R.

In paper 2, density was assessed for stromal CXCL16 and tumor cell CXCR6 and scored in the following manner 0 = no cells showing positivity, 1 = less than 25 % positivity, 2 = 25-50 % positivity, and 3 = 50-100 % positivity. This score was added to the intensity score. High tumor cell CXCR6 was

defined as a score ≥5.5, high stromal cell CXCL16 was defined as a score ≥3. CXCL16 in tumor cells was scored according to intensity only, with a high score defined as ≥2.5.

In paper 3, the Tromsø cohort was used as a training set, with the following scoring cutoffs for stromal CD8 density: 0-5% = 0, 5-25% = 1, 25-50% = 2 and > 50% = 3. Only two patients in the training set had an average or maximum of 0, consequently score 0 and 1 were merged. The following cutoffs were therefore examined in validation sets: Low density: ≤ 25%; intermediate density: >25% ≤ 50%; high density: > 50%.

In paper 4, LAG-3 expression on immune cells was assessed in the stromal and intraepithelial compartments in primary tumors. In the metastatic lymph nodes, the intraepithelial and extraepithelial were scored. In the intraepithelial compartments the number of intraepithelial LAG-3 + cells were counted in each core and scored according to the following scale: 0 = absent, 1 = 1-9, 2 = 10-50, 3 = >50. In stromal and extraepithelial compartments LAG-3 was scored according to the percentage of immune cells displaying positivity: 0 = absent, 1 = 1-24%, 2 = 25 -50%, 3 = > 50%. A high score was defined as mean core score of > 0 for intraepithelial compartments, and >0.5 for stromal and extraepithelial compartments.

3.5 Cutoff determination

Mean values for the duplicate stromal and tumor tissue cores were calculated and used to determine cutoffs for dichotomization in papers 1,2 and 4. For markers that were scored according to density and intensity, the mean values were summed before dichotomization. In paper 3, the maximum score for each patient was used, as it resulted in the optimal significant prognostic impact. For all the paper in this thesis cutoff were selected using a minimum p-value approach; i.e. dichotomizing makers based on the lowest value of the log-rank test statistic. The cutoff chosen is thus the one which best differentiates the two groups according to disease-specific survival (DSS).

3.6 Statistical methods

Statistical analyses were performed using using the statistical package IBM SPSS, versions 20-24. (IBM Corp., Armonk, NY, USA) and Rstudio 1.0.44 (RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, USA) with the libraries “survival” [144], “ggplot2” [145] and “survminer” [146]. Two sided P-values < 0.05 were considered statistically significant. We used DSS, defined as the time from surgery to death of lung cancer, as the primary endpoint. Additionally, OS, defined as time from surgery to death of any cause, and disease-free survival (DFS), defined as time from surgery to first lung cancer recurrence, were examined as secondary endpoints. Correlations between molecular markers were calculated using Spearman’s rank correlation analysis. Relationships between clinicopathological variables and molecular marker expressions were assessed with the $\chi 2$ or Fischer exact test. The Kaplan-Meier method was utilized in univariate survival analysis of clinicopathological variables and molecular markers. Statistical significance of differences between survival curves was assessed with the log-rank test. For multivariate analysis the Cox Proportional Hazards model was used utilizing the backward conditional method for model fitting. Probability for stepwise entry and removal set at 0.05 and 0.10, respectively. Significant variables from univariate analysis were entered into the multivariate model.

The original sample size calculations performed for this study indicated that 300 subject were needed to achieve a power of 80% at an alpha of 5% (PASS 2002, Number Cruncher Statistical Systems, Kaysville, Utah, USA). This calculation used DSS as the primary endpoint, presumed that a 50% increase in hazard from a given marker represented a clinically significant effect, that the 5-year DSS of NSCLC is approximately 60% and that the frequency of the given marker was 35% [147].

3.7 RNA interference and proliferation assay

In paper 2, we used siRNA technology to study the effects of CXCL16 expression on cell proliferation. RNA interference (RNAi) is a conserved biological response to double-stranded RNA which inhibits gene expression, typically by causing destruction of specific mRNAs. Small interfering RNA (siRNA) are the main effectors of RNAi, and synthetic siRNA can be constructed to knock down the expression of a specific gene [148]. The commercial NSCLC cell lines utilized were NCI-H460 (Large cell carcinoma) and A549 cells (adenocarcinoma). To monitor the effect of the siRNA we employed the xCelligence RTCA DP system (Roche). By measuring impedance thru micro-electrodes on the bottom of tissue culture plates, the system allows for real time assessment of cell numbers and doubling time.

3.7.1 Assay procedure

The detailed experimental procedures for the siRNA experiments, including cell culture conditions and reagents, are described in paper 2. In short, the cells were typsinized briefly for detachment, before resuspension in growth media and counting. They were then seeded as duplicates into E-plates, which were incubated in room temperature for 1 hour before transfer to the xCelligence instrument. Six hours post seeding the siRNA transfection mix was added, and after further 4 hours replaced with regular growth media. The cell index (the unit of the cell-sensor impendance) was measured every 15 minutes for the first 4 hours of the experiment, subsequent measurements were taken every 30 minutes.

3.8 Ethics

Study approval was obtained from the respective regional ethical committees (Tromsø and Bodø: protocol ID: 2011/2503; Oslo: protocol ID 2009/1904; Denmark: protocol ID: 20080018).

4. MAIN RESULTS

4.1 Patient characteristics

Table 2 gives an overview of the clinicopatological characteristics of the different patient cohorts examined in this thesis. Detailed tables for the separate cohorts and the influence of clinicopathological variables on survival are presented in the respective papers.

Table 2. Clinicopathological characteristics for the separate cohorts examined in each paper (1-4)

	Paper 1	Paper 2	Paper 3, training cohort	Paper 3, validation cohort	Paper 3, validation cohort.	Paper 3, validation cohort	Paper 4
Hospital	UNN + NH	UNN + NH	UNN	NH	OUS	OUH	UNN + NH
Number of patients	55	335	155	169	295	178	553
Time of inclusion	1990–2005	1990–2005	1990–2005	1990–2005	2006–2011	1992–1999	1990–2010
Median age	65 (39–76)	67 (28–85)	67 (39–85)	67 (28–82)	67 (39–84)	64 (39-82)	67 (28–85)
Last follow–up	Jan 2011	Jan 2011	Jan 2011	Jan 2011	March 2014	Jan 2010	Oct 2013
Median follow–up of survivors (months)	113 (73–197)	105 (72–234)	111 (6–222)	103 (73–234.0)	52 (35–99)	162 (121– 211)	86 (34–267)
Median DSS (months)	44	190	121	190	NR	116	190
Median DFS (months)	44	119	80	178	87	92	68
Median OS (months)	24	45	47	42	79	50	47
5-year DSS (%)	44	58	57	58	69	57	58
5-year DFS (%)	46	55	51	57	61	53	52
5-year OS (%)	33	44	45	42	60	47	45

Hospitals (city, country): UNN = University hospital of North Norway (Tromsø, Norway); NH = Nordland Hospital (Bodø, Norway); OUS = Oslo University Hospital (Oslo, Norway); OUH Odense University Hospital (Odense, Denmark).

4.2 Paper 1

Radiotherapy may stimulate the anti-tumor immune response and radiotherapy and immunotherapy can have synergistic effects. We explored the prognostic significance of different immune cell markers in a subgroup of NSCLC (N=55) patients treated with postoperative radiotherapy (PORT).

4.2.1 Univariate analysis

Tumor epithelial and stromal expression of CD1a+, CD3+, CD4+, CD8+, CD20+, CD56+, CD68+, CD117+ and CD138+ cells, as well as M-CSF and CSF-1R, was assessed by immunohistochemistry. High expression of CD4 (P <0.001) and CD1a (P=0.025) were significantly associated with an improved DSS. A combined high stromal expression of CD4 and CD8 was also a positive factor for DSS (P <0.001).

4.2.2 Multivariate analyses

Compared to patients with a combined high stromal CD4 and CD8 expression, the patients with a combined low CD4 and CD8 expression had a hazard ratio of 21.2 (95% CI: 4.5 - 120.4, P < 0.001), while the other CD4/CD8 combinations had a hazard ratio of 1.8 (95% CI: 0.4-8.4, P = 0.430). Low stromal CD1a expression had a hazard ratio of 2.5 (95% CI: 0.97 – 6.2, P = 0.058) compared to high expression.

4.3 Paper 2[*]

The chemokine CXCL16 and its receptor CXCR6 are expressed on a variety of immune cells, and has been significantly correlated with prognosis in various cancers. We explored their immunohistochemical expression and prognostic impact in a cohort of 335 NSCLC patients. The utilized antibodies were validated by western blots and siRNA knockdown. We examined the effects of CXCL16 on cell proliferation by performing siRNA mediated knockdown in two NSCLC cell lines.

4.3.1 Expression and correlations

CXCL16 was expressed both in stromal and cancer cells, whereas CXCR6 expression was only detected in cancer cells. The stromal cells displaying positivity for CXCL16 were fibroblasts, endothelial cells, macrophages and plasma cells. There were no significant correlations between CXCL16 or CXCR6 and innate (CD68m CD56, CD1a) or adaptive (CD4, CD8, CD3, CD20) immunological markers. Stromal and cancer cell CXCL16 correlated significantly ($r = 0.368$, $P < 0.01$), whereas the expressions of CXCR6 and CXCL16 in cancer cells did not.

4.3.2 Univariate analysis

High expression of CXCL16 in stromal cells was associated with improved DSS ($P = 0.016$). Additionally, the combination of high stromal CXCL16 and high cancer cell CXCL16 was associated with an improved DSS ($P = 0.016$). Neither cancer cell CXCL16 nor CXCR6 had significant association with DSS in univariate analyses.

4.3.3 Multivariate analysis

Stromal CXCL16 and the co-expression variable of stromal and cancer cell CXCL16 were entered into two separate multivariate analyses, together with significant clinicopathological variables. In the first model, high expression of stromal CXCL16 was an independent positive prognosticator (HR: 0.55; 95% CI: 0.35 -0.87, $P = 0.011$). Similarly, combined high expression of CXCL16 in cancer and stromal cells was an independent prognostic factor for an improved DSS (HR: 0.42; 95% CI: 0.20-0.88, $P = 0.022$), when compared to combined low expression.

4.3.4 Cell proliferation

Utilizing the xCELLigence platform, we observed that knockdown of CXCL16 with siRNA caused increased proliferation compared to the negative scrambled control ($P < 0.001$). Similar effects were observed in two different NSCLC cell lines: A549 and NCI-H460.

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[*] Table 2 and Table 3 for this paper contain some errors, as the first author erroneously supplied an early draft of these tables to the journal editorial office. The correct versions of the tables are appended at the end of paper 2.

4.4 Paper 3

Evidence suggest that *in situ* immune cell infiltrates can be prognostic in many cancers, including NSCLC. We have previously investigated various immune cell markers in NSCLC, with stromal CD8 + tumor infiltrating lymphocytes (TILs) as the most promising markers [116]. Hence, we aimed to validate the prognostic impact of stromal CD8 TILs in four different cohorts from Norway and Denmark, compromising a total of 797 NSCLC patients.

4.4.1 Univariate analysis

In the total material, stromal CD8+ density had significant prognostic impact using three different endpoints: DFS (P < 0.001), DSS (P < 0.001), and OS (P < 0.001). The corresponding 5-year survival rates for high, intermediate and low score were 68%, 59%, and 43% for DFS; 74%, 63%, and 49% for DSS; and 61%, 50%, and 41% for OS. For each separate cohort, the prognostic impact using DSS as the endpoint was as follows: Tromsø (P = 0.004), Bodø (P = 0.242), Oslo (P = 0.295), and Odense (P = 0.009). Using DSS as the endpoint, CD8+ density had significant prognostic impact in all subgroups when stratified by histology and pathological stage. In the Danish cohort, analysis of CD8+ density was also stratified by the location of the tissue cores (central tumor versus the invasive margin). With DSS as the endpoint, the prognostic impact of stromal CD8+ density was highly significant at the invasive margin (P = 0.008) but not in the central tumor (P = 0.67).

4.4.2 Multivariate analysis

Pathologic stage, tumor differentiation, and stromal CD8+ density were independent prognostic factors for all endpoints. With DSS as endpoint, HR was 1.48 (95% CI: 1.05 – 2.09, P = 0.026) for intermediate and 2.31 (95% CI: 1.61 – 3.31, P = 0.001) for low, when compared to the reference of high stromal CD8 + density (P <0.001 overall significance as a prognostic factor).

4.5 Paper 4

LAG-3 is in immune checkpoint molecule involved in the negative regulation of T-cell responses. We sought to investigate the prognostic impact of LAG-3 in resection specimens from 553 NSCLC primary tumors and 143 metastatic lymph nodes and asses potential for inclusion in NSCLC Immunoscore.

4.5.1 Expression and correlations

LAG-3 displayed a homogenous membranous/diffuse cytoplasmic staining, and its expression was confined to tumor-infiltrating lymphocytes (TILs). The expression of LAG-3 in stromal and intraepithelial compartments was significantly correlated (r=0.63, P<0.001), as was the expression in intraepithelial and extraepithelial compartments in the metastatic lymph nodes (r=0.60, P<0.001). LAG-3 expression was strongly correlated to multiple immune cell markers including CD8, CD3, CD4 and PD-1.

4.5.2 Univariate analysis

Expression of LAG-3 in intraepithelial (P=0.003) and stromal (P<0.001) compartments in primary tumors was associated with an improved DSS. In the metastatic lymph nodes, expression of LAG-3 in the intraepithelial and extraepithelial compartments was associated with improved DSS. Neither intraepithelial nor stromal LAG-3 in primary tumors had significant prognostic impact across all pathological stages, and combining their expression with other immunological markers did not improve patient stratification according to DSS.

4.5.3 Multivariate analysis

In a multivariate model were stromal and intraepithelial LAG-3 were assessed with stromal CD8, intraepithelial CD45RO, stromal PD-1+tumor PDL-1 and clinicopathological variables, stromal LAG-3 remained an independent predictor of improved DSS (HR 0.59, 95%CI 0.43-0.82, P=0.002). Both intraepithelial (HR 0.61, 95% CI 0.38-0.99, P=0.049) and extraepithelial (HR 0.54, 95% CI 0.29-0.70, P<0.001) LAG-3 in the metastatic lymph nodes were associated with an improved DSS in multivariate models.

5. DISCUSSION

5.1 Methodological considerations

5.1.1 Patient cohorts

A strength of this thesis is that the included patients in our primary cohort compromises approximately 90% of all operated NSCLC patients in our region during the inclusion period, reducing the risk of selection bias. Patients who received neoadjuvant treatment or were diagnosed with a separate malignancy within 5-years of lung cancer diagnosis were excluded. This was done as neoadjuvant therapy may affect the lung cancer microenvironment (e.g. attract immune cells), similarly a previous malignancy may affect the response to a new primary malignancy.

Our results may potentially be confounded by the long inclusion time, as there have been changes in post-surgical treatment over time, notably with the introduction of adjuvant chemotherapy in 2005. Additionally, our database lacks information on how clinical staging has been performed. As the clinical staging improves (e.g. because of PET-scans), there is a risk of stage migration. Consequently, the survival may improve as more cases of advanced (i.e. inoperable) disease is recognized as advanced rather than limited disease, and therefore not included in the dataset [149].

We chose DSS as our primary endpoint in this study. Many patients with lung cancer are elderly and suffer co-morbidities; consequently, they may die of other causes than lung cancer. We believe DSS may be more relevant endpoint than OS when investigating a novel prognostic marker. This way, we may more specifically relate marker expression to the underlying biology of the disease. There exists a risk that using DSS as an endpoint may result in some misclassifications, however, we critically reviewed all cases according to patient files. We also examined DFS was as an endpoint, but as this was a retrospective study and the follow-up of the patients was not standardized, DFS estimates are potentially less accurate. The retrospective nature of this study has also limited our ability to collect a detailed smoking history for the patients. For the same reason, we were unable to explore the influence of relevant and interesting comorbidities such as COPD and autoimmune diseases.

5.1.2 Histopathology and staging

The histological and pathological classification of NSCLC in constantly evolving. Accordingly, we have updated our database during the course of this study as changes were implemented. Thus, paper 4 differs from the other papers with regards to histology and TNM classification. While this makes direct comparisons between the studies somewhat more difficult, we nevertheless believe it is important to use the classifications that reflect the current scientific consensus. A weakness of our

database is that it lacks information on key driver mutations (e.g. EGFR, KRAS, ALK), which could be highly relevant in relation to both patient prognosis and marker expressions.

5.1.3 TMA: Advantages and disadvantages

TMAs have several advantages when compared to conventional histological slides. If not using TMAs, histologic sections would have to be cut individually from potentially hundreds of separate paraffin blocks. These sections then would need to be stained in multiple successive procedures, before being subject to evaluation by a pathologist to identify areas of tumor and asses the level of staining. When investigating multiple markers, this time consuming process must be fully repeated. In contrast, after construction, TMAs facilitate the rapid analysis of multiple markers using only one slide from each TMA block per investigated marker. As the tissue cores included in a TMA are selected by an experienced pathologist, scoring of a stained biomarker can often reliably be done by a non-pathologist [135]. The limited number of sections needed for staining has an important advantage in reducing experimental variability, as all cases and controls can be stained under the same experimental conditions. TMAs also have the obvious benefit of preserving original tissue, as only small cores are used from each donor block, amplifying the number of assays that can be performed per archived sample by up to a thousandfold [135]. Additionally, TMAs facilitates the sharing of samples between research laboratories.

Ever since the introduction of TMA technology, concerns have been raised about the small diameter of the tissue cores. Tissues, and especially tumors, are commonly heterogeneous and different parts and cells of a tumor may display significant genetic differences which can be difficult to recognize on 0.6 mm tissue spots [150]. Nevertheless, it should be noted that conventional whole sections have similar issues regarding representativity; for a tumor with a diameter measured in centimeters a conventional tissue section will only ever represent a fraction of the total tumor volume [151]. While some information may be lost when using TMAs compared to whole sections, the sampling error is diluted when a large cohort is analyzed [135]. A number of studies have validated the TMA method by reproducing well recognized associations between biomarker expression and patient prognosis [151-153]. In NSCLC, acceptable agreement has been demonstrated between whole sections and TMA when comparing well characterized markers in tumor cells [154-156]. While two 0.6 mm tissue cores often adequately represent whole sections, the concordance between whole sections and TMAs may vary between different markers and tissues [135,142].

Table 3. Advantages and disadvantages on TMAs	
Advantages	**Disadvantages**
Time saving, high-throughput method	TMA construction can be challenging and requires technical expertise
Increased utilization of limited tissue resources	Validity may be reduced for heterogeneously expressed biomarkers
Reduced experimental variability	Loss of tissue cores on cut sections may reduce statistical power
Scoring by non-pathologists feasible	Tumor content may change through the length of a tissue core
Ease of sharing with other laboratories	Not validated for individual diagnosis
Decreased experimental costs and assay volume	

5.1.4 Immunohistochemistry

Adequate fixation is a prerequisite for optimal staining results by IHC. Since this study is retrospective, variations in fixation times and other preanalytic factors over time and between the different hospitals may have influenced our results. Additionally, there is a concern that long-term storage may affect antigenicity even of adequately fixated tissues [157]. However, even 60-year paraffin-embedded specimens have been shown to retain their antigenicity for commonly used diagnostic markers [158,159]. Reassuringly, we have not seen significant differences in staining intensities and distributions between older and newer samples for our examined markers. In paper 3, the staining distribution for the four hospitals was largely similar.

5.1.5 Cutoff selection and scoring

For most biomarkers (including those examined in this thesis) a common reference standard for scoring is lacking, leaving the method of scoring and cutoff determination at the choice of the investigators, which in turn can make comparing results from different studies challenging [160]. While numerous different scoring strategies for IHC exist (e.g. Allred, IRS and H-score), none of them have been validated for the markers covered in this thesis. We examined all markers using a semi-quantitative approach similar to what we have used in previous studies [116,161]. This method is simple, pragmatic, and time-efficient, and facilitates comparisons with our previously investigated markers.

In the papers presented in this thesis, we have chosen cutoffs by a minimum p-value approach. As our approach has been explorative and hypothesis generating, we consider this approach is

appropriate. A danger of choosing an optimal cutoff it that it increases the risk of false positive results (type 1 error) [162]. Choosing the mean or median as a cutoff reduces this risk, however it may not be the cutoff that best describes the underlying biology of the marker, which may result in false negative results (type 2 error). Regardless of the choice of cutoff, replication of results in an independent cohort is necessary for validation of a putative prognostic marker. In paper 3, we were able to investigate one of our markers in this manner, using a part of our initial cohort (the UNN, Tromsø cohort) as a training set for cutoff determination and then examining this predefined cutoff in three validation sets.

5.2 Discussion of main results

5.2.1 The *in situ* immune infiltrate in patients treated with adjuvant radiotherapy.

In this study, we evaluated a number of innate and adaptive immune markers in NSCLC patients treated with PORT, and found that a combined low density of CD4 and CD8 in the stromal compartment was associated with a dismal DSS. Both increased CD4 and CD8 have previously been shown to be linked to improved prognosis in NSCLC [116,163], though to our knowledge, no other studies have examined their prognostic impact in NSCLC patients treated with PORT.

This study has several limitations, the most important being the low sample size. Therefore, the obtained results must be interpreted with caution, as a limited number of events contribute to the observed hazard ratios. Additionally, some patients in this study received PORT for N1 disease, which is now known to adversely affect prognosis [112]. Norwegian guidelines currently recommend PORT for patients with tumor-positive resection margins and those with N2 disease [35]. However, the use of PORT in N2 disease remains controversial and will be clarified by ongoing clinical trials [112].

In head and neck cancer, a retrospective study of 101 patients undergoing definitive chemoradiotherapy, high levels of intraepithelial CD3 and stromal CD8 cells were found to be indicative of improved survival [164]. It is not possible to tell if the improved survival seen in patients with increased T-cell infiltrates represent an increased sensitivity to radiotherapy. However, multiple pre-clinical studies show a close connection between the immune system and the effects of radiotherapy. Studies in mice have shown that effective ablative radiotherapy is at least partly dependent on T-cells [165]. Tumor cell death induced by radiotherapy causes release of multiple molecules in the tumor microenvironment, which in turn can lead to activation of dendritic cells and priming of effector T-cells [166]. Radiotherapy can also enhance the diversity of the T-cell receptors expressed by intratumoral T-cells [167]. While radiotherapy can induce T-cell responses, it can also stimulate recruitment of immunosuppressive macrophages [168]. Indeed, the immunosuppressive microenvironment present in most cancers may mask pro-immunogenic effects of radiotherapy [169]. It is therefore tempting to speculate that an increased adaptive immune infiltrate represents a less immunosuppressive tumor microenvironment more receptive to radiotherapy. Combining radiotherapy with various forms of immunotherapy has shown efficacy in multiple pre-clinical models of cancer [115]. Multiple clinical trials evaluating the effect of combining these treatment modalities are currently underway in NSCLC [170] .

Multiple studies have shown that an increased infiltration of TILs (CD8+ cells in particular) are associated with improved outcomes in NSCLC [116,171]. Stereotactic radiotherapy is increasingly used as a curative treatment modality in inoperable early stage NSCLC [172]. Because of the noted link between

radiotherapy and immune responses, it has recently been suggested that the prognostic impact of the immune infiltrate should be considered also in patients treated with stereotactic radiotherapy [173]. In light of the results presented in our study, we suggest that this evaluation should also be carried out in future trials of PORT in NSCLC.

5.2.1 CXCL16 and CXCR6

This study demonstrated that high expression of stromal CXCL16 was an independent positive prognosticator (HR: 0.55; 95% CI: 0.35 -0.87, P = 0.011), as was the combined expression CXCL16 in cancer and stromal cells (HR: 0.42; 95% CI: 0.20 -0.88, P = 0.022). Our results were supported in cell experiments, where knockdown of CXCL16 with siRNA caused increased proliferation.

While CXCR6 and CXCL16 expression has been observed in many human cancers, only a limited number of studies have investigated their impact on prognosis. Of these studies, none include independent validation cohorts, making it difficult to reach firm conclusions regarding outcome. However, results indicate that CXCR6 and CXCL16 has disparate impacts on survival in different malignancies, reflecting the multifaceted roles of these chemokines in cancer biology. In colorectal cancer, high levels of CXCL16 in tumor tissues have been correlated with a worse prognosis [123]. This was linked to increasing levels of infiltrating lymphocytes CD4+ and CD8+ lymphocytes [123]. However, in our cohort we found no correlation between CXCL16 or CXCR6 and markers of lymphocytes and other immune cells. In gastric cancer, nuclear CXCL16 expression was linked to improved survival in univariate analysis [174]. In renal cancer, a study of 104 resection specimens found increased CXCL16 expression to be correlated with improved survival [124]. Using siRNA to silence CXCL16 expression, reduced migration of renal cancer cell lines was noted. Normal lung and renal tissue both constitutively express CXCL16. Thus, loss of or aberrant CXCL16 expression may play a role in cancer development in these tissues, however, this remains to be confirmed experimentally. Experimental evidence also suggests other mechanisms for an anti-tumor effect of CXCL16 and CXCR6 in cancer development. In CXCR6 knockout mice, liver metastasis from Lewis lung carcinoma cells was increased compared to wild-type mice [175]. Similarly, liver metastasis increased in wild-type mice treated with an anti-CXCL16 antibody [175] .

Increased expression of CXCL16 and CXCR6 in prostate cancer [176] and CXCR6 [121] in liver cancer has been linked to poor prognosis. Notably, our research group has analyzed CXCR6 and CXCL16 expression in prostate cancer, utilizing the same antibodies and TMA technology as in the present NSCLC study. In a multivariate analysis of 535 patients, increased levels of CXCL16 and CXCR6 were independent predictors for clinical failure in multivariate analysis [176]. Pre-clinical evidence in prostate cancer models support this finding, where the CXCL16-CXCR6 axis has be linked to increased cancer cell invasiveness and bone metastasis [122,177].

To our knowledge, ours is the only study that has investigated the prognostic impact of CXCL16 and CXCR6 in NSCLC. However, other studies have also noted their expression in NSCLC resection specimens. Hu and colleagues analyzed CXCR6 expression in 33 NSCLC patients and found both CXCR6 and CXCL16 to be expressed in the majority of the examined tissues [178]. In cell line studies, they found that soluble CXCL16 or conditioned medium from NSCLC cell lines could improve the *in vitro* viability and invasiveness of NSCLC cell lines. Similar results shown by Mir and colleagues [179], may suggest that CXCL16 has different effects in NSCLC depending on whether it exists in soluble or transmembrane form. In ovarian cancer, elevated CXCL16 in patient serum was found to be an independent predictor of poor survival [180]. While elevated serum CXCL16 has been noted in NSCLC patients compared to normal controls [179], the prognostic impact of this finding in NSCLC remains to be elucidated.

5.3.1 Stromal CD8

In our analysis of stromal CD8 in 797 NSCLC patients, we found that stromal CD8+ TIL density was a strong independent prognostic factor for DFS, DSS and OS for the patient material analyzed as a whole. While the prognostic impact was not statistically significant for all endpoints in all cohorts, we consistently observed a clear trend for improved survival for higher levels of stromal CD8.

A major strength of this study is the large sample size. Additionally, the examined cutoffs were predetermined for the validation cohorts. When the TMA slides from Denmark were stained and reevaluated with the same IHC procedure as that used in the Oslo cohort, we found a significant correlation with the original staining scores and a similar impact on survival. Thus, the different staining procedures appear to have little impact on our results, furthermore the scoring of stromal CD8 is reproducible between different scorers. The different diameters used for the tissue cores in the different TMAs (0.6mm vs 1mm) may be a weakness in this study. However, we observed similar trends relating to survival regardless of core diameter.

The prognostic impact of TILs has been investigated in numerous retrospective studies in NSCLC, the majority of these studies have also analyzed the impact of CD8+ T-cells specifically [100]. In a 2015 meta-analysis (where this study was included), both intraepithelial (cancer nest) and stromal CD8 infiltration was associated with improved overall survival in NSCLC [104]. However, stromal CD8 has previously been found to have the strongest prognostic impact in our cohort [116], and we therefore chose to analyze it further in the present study. In the Danish cohort, the positive prognostic impact of stromal CD8 was restricted to tissue cores taken from the tumor invasive margin. Unfortunately, we were unable to explore this finding in the other cohorts, as the sample location for the tissue cores was not recorded when these TMAs were constructed. However, the cores in the Norwegian cohorts are a mix of cores from the central tumor and tumor periphery. Hence, we assume that for

most patients a core from the invasive margin is in fact present, though the exact frequency is unknown. This may in turn contribute to explaining why the maximum CD8 density (not the mean) for each patient had the most significant impact on survival.

Stromal CD8 seems to be a promising prognostic marker in NSCLC, as it has significant prognostic impact within each TNM stage, is reproducible, and is simply and readily detectable in patient samples. However, in order to be translated into the clinic, numerous issues must be addressed. While TMAs are an important research tool for biomarker discovery in large cohorts, their suitability for individual patient diagnostics remain unknown. Further, the best and most accurate method for quantification of immune cells (including CD8+ cells) in the tumor microenvironment in NSCLC remains unknown. Though CD8 has been associated with prognosis in numerous studies, significant heterogeneity exists between studies with regards to sampling (TMA vs whole sections, tumor nest vs stroma) and quantification (absolute count vs percentages, small vs. larger field views). A recent analysis investigated CD8 sampling strategies in 23 NSCLC resection specimens, comparing eight different sampling strategies to an absolute, automated CD8 count in whole sections [181]. They found significant correlations between all eight sampling strategies and whole tumor counts; however, the most concordant results were derived from random sampling of 20 % of the tumor, a simulated core biopsy, or from sampling the tumor center. They found CD8 infiltration to be associated with survival when sampling the tumor center ($p = 0.038$), but not the invasive margin ($p > 0.2$) which contrasts with our findings in the Danish cohort. However, this study supports that estimation of CD8 in small samples (such as biopsies) allow for reasonably accurate estimates of CD8 counts in whole sections, though the sample size (*n* =23) of this study is small. Thus, the estimation of stromal CD8 might be feasible also for patients with unresectable NSCLC, though the prognostic impact in this patient group remains unknown.

The scoring performed in our present study is subjective. The use of a three-tiered scale may be unsuitable in clinical practice, and a two-tiered scale will be considered in future studies. An approach incorporating digital pathology may allow for a CD8 enumeration that is more objective than manual scoring. Schalper and colleagues, who identified CD8+ TILs as an independent prognostic marker in NSCLC by multiplexed quantitative fluorescence, have shown the feasibility of such an approach on TMAs [182].

In addition to stromal and intraepithelial CD8, multiple other immunological markers have potential for inclusion in an NSCLC immunoscore to supplement the TNM. A recent large trial incorporating a discovery set (one trial, 824 patients) and a validation set (three trials, 984 patients) found that increased TIL intensity as evaluated by morhoplogical criteria on H&E sections was significantly

associated with prognosis [171]. Similar results have been observed our cohort of 553 NSCLC patients (M. Khanehkenari, personal communication). However, analyzing specific TIL subsets (such as CD8 and CD45RO) and tumor compartments may enhance the prognostic value of TIL analysis in NSCLC.

5.3.2 LAG-3

In our cohort of 553 NSCLC patients, we found LAG-3 expression on TILs in both primary tumors and metastatic lymph nodes to be independently associated with improved survival.

To our knowledge, this is the first study to investigate the prognostic impact of LAG-3 expression in metastatic lymph nodes. Interestingly, we found a significant prognostic impact of LAG-3 expression both in the intraepithelial and extraepithelial compartments. The impact of LAG-3 expression in the extraepithelial compartments was stronger and more significant than for the intraepithelial compartment, presumably because of some of examined cores lacked sufficient malignant epithelium and consequently not scored for intraepithelial LAG-3. A strength of our findings is the fact that the same cutoffs as previously established in the primary tumors could be successfully employed in the metastatic lymph nodes.

A limited number of studies have examined the prognostic impact of LAG-3 expression in resection specimens using immunohistochemistry. In renal cancer, increased LAG-3 expression at the invasive tumor margin in a group of patients with increased CD8 infiltration (n=40) was associated with shorter overall survival [183]. In head and neck cancer, increased LAG-3 expression on TILs was negatively associated with survival in a subgroup analysis of 78 patients without lymph node metastasis [184]. However, a larger study of 402 patients found LAG-3 expression to be associated with improved survival, although this finding was not statistically significant [185]. In a NSCLC retrospective study of 139 patients, increased LAG-3+ TILs were associated with a worse prognosis in univariate analysis, however, this finding was not significant in the multivariate analysis [130]. Similar to what we observed in our cohort, this study found LAG-3 expression on TILs in resection specimens. However, their results concerning outcome contrasts with ours. The longer follow-up and sample size in our study, in addition to methodological differences between the studies, may contribute to explaining this discrepancy.

Immune checkpoints such as LAG-3 and PD-1 appear to negatively regulate T-cell responses, however expression of immune checkpoints on TILs do not necessarily correlate with an adverse prognosis in cancer. In early stage NSCLC, analyses of TILs from 87 resection specimens have shown that TILs expressing LAG-3 and other immune checkpoint molecules display a recently activated phenotype and remain functional [186]. In melanoma, expression of LAG-3 together with other immune checkpoints has been shown to identify tumor-reactive and mutation specific T-cells [187]. Thus, we

hypothesize that LAG-3 expression in resection specimens represents patients who have an ongoing immune response to tumors. Apparently, this immune response has been insufficient, as the tumor has been able to grow to a size necessitating its surgical removal.

We do not consider LAG-3 expression in primary tumors to be a good candidate for an NSCLC Immunoscore, as it lacked prognostic impact in all pathological stages and did not improve patient stratification when combined with other candidate markers. However, LAG-3 in metastatic lymph nodes should be explored in future studies, as it prognostic impact was evident in both the intraepithelial and extraepithelial compartments. Future studies should also consider the influence of LAG-3 expression in different TIL subsets, as well as the expression of putative LAG-3 ligands LSECTIN and Galectin-3.

6. CONCLUSIONS

This thesis includes four different studies of immune-related markers in NSCLC. While the patient groups and markers differ between these studies, the aims and methods are similar; we attempt to characterize a part of the immunological landscape in NSCLC and its relation to prognosis. In the first study, we present analyses of multiple adaptive and innate immune markers in resected NSCLC treated with radiotherapy, and find increased CD4/CD8 expression to be associated with a markedly improved DSS. While the sample size is small, these results are intriguing in light of the increasing evidence of radiotherapy influencing immune responses. As there is no current molecular marker to predict response and prognosis in relation to radiotherapy in NSCLC, it would be interesting to explore this relation further. This could be done in patients who receive PORT, but also in those treated with stereotactic radiotherapy.

Cytokines and chemokines contribute to shaping the tumor microenvironment and the immune contexture. In paper two, we characterized, for the first time, the expression and prognostic impact of the chemokine pair CXCR6 and CXCL16 in NSCLC. While neither chemokine had a significant correlation with other investigated markers, loss of CXCL16 expression was associated with a worse prognosis, which suggests it may have some role in NSCLC development. In this study, we were also able to expand our tissue-based findings with cell proliferation assays. Exploring the mechanisms behind tissue based findings is an important, though complex, part of expanding our knowledge on the pathophysiology of NSCLC and the immune system's role in in its development.

In the fourth study, we explored the prognostic impact of LAG-3, a novel target of immunotherapy in NSCLC. We were also able to include analysis of metastatic lymph nodes and to show that even for patients with apparently more aggressive disease (i.e. confirmed lymph node metastasis), expression of LAG-3 can identify patients with improved prognosis. While our findings of improved survival with LAG-3 expression may seem counterintuitive, we suggest that its expression suggest an active, yet insufficient, immune response to tumors. Indeed, the argument can be made that any patient who requires surgery for a malignant tumor has been through an insufficient immune response. However, for some patients the immune response may be sufficient to halt metastatic spread and local recurrence following surgery. Nevertheless, any prognostically favorable immune infiltrate could be a secondary phenomenon, i.e. that it represents an underlying tumor biology that by itself is less aggressive and less likely to metastasize. Regardless of the mechanisms behind the prognostic impact of the immune contexture in NSCLC, characterizing its components has a potentially important prognostic utility.

In contrast to the explorative approach in the other studies in this thesis, the third study aimed to validate stromal CD8 as a prognostic marker in several NSCLC cohorts. Using cohorts from four different hospitals, we saw a consistent impact of stromal CD8 with regard to survival for all survival endpoints. Strikingly, stromal CD8 had significant prognostic impact across all pathological stages in the total patient material, and was independently associate with an improved prognosis in multivariate analysis. Thus, we suggest stromal CD8 to be a promising candidate marker for an NSCLC immunoscore. A prospective trial has now been initiated in order to validate an immunoscore for NSCLC, with stromal CD8 as one of the included markers. If successfully validated for a prognostic impact, future trials may also consider the predictive value of a NSCLC immunoscore. Conceivably, this could improve the patient selection for adjuvant treatment, ultimately improving survival for NSCLC patients.

7. REFERENCES

1. GLOBOCAN 2012 v1.0, Cancer incidence and mortality worldwide: IARC CancerBase No. 11 Lyon, France: International Agency for Research on Cancer; 2013. (Accessed 10.01.2017, 2016, at http://globocan.iarc.fr.)
2. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Research 2014;74:2913-21.
3. Brustugun OT, Moller B, Helland A. Years of life lost as a measure of cancer burden on a national level. British Journal of Cancer 2014;111:1014-20.
4. Cancer Registry of Norway. Cancer in Norway 2015 - Cancer incidence, mortality, survival and prevalence in Norway. Oslo: Cancer Registry of Norway; 2016.
5. Lund KE, Lund M, Bryhni A. Tobacco consumption among men and women 1927-2007. Tidsskrift for den Norske laegeforening 2009;129:1871-4.
6. Dela Cruz CS, Tanoue LT, Matthay RA. Lung Cancer: epidemiology, etiology, and prevention. Clinics in Chest Medicine 2011;32:10.1016/j.ccm.2011.09.001.
7. Alberg AJ, Samet JM. Epidemiology of lung cancer. Chest 2003;123:21S-49S.
8. Loeb LA, Emster VL, Warner KE, Abbotts J, Laszlo J. Smoking and lung cancer: an overview. Cancer Research 1984;44:5940-58.
9. Warren GW, Cummings KM. Tobacco and lung cancer: risks, trends, and outcomes in patients with cancer. American Society of Clinical Oncology educational book. American Society of Clinical Oncology Meeting 2013:359-64.
10. Sun S, Schiller JH, Gazdar AF. Lung cancer in never smokers--a different disease. Nature Reviews Cancer 2007;7:778-90.
11. Straif K, Cohen A, Samet J. Air Pollution and Cancer (IARC Scientific Publication No. 161) International Agency for Research on Cancer, Lyon, France 2013.
12. Alberg AJ, Brock MV, Ford JG, Samet JM, Spivack SD. Epidemiology of lung cancer: Diagnosis and management of lung cancer, 3rd ed: american college of chest physicians evidence-based clinical practice guidelines. Chest 2013;143:e1S-e29S.
13. Rushton L, Hutchings SJ, Fortunato L, et al. Occupational cancer burden in Great Britain. British Journal of Cancer 2012;107 Suppl 1:S3-7.
14. Novello S, Barlesi F, Califano R, et al. Metastatic non-small-cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of Oncology 2016;27:v1-v27.
15. Travis WD. Pathology of lung cancer. Clinics in Chest Medicine 2011;32:669-92.
16. Tomashefski JF, Jr., Connors AF, Jr., Rosenthal ES, Hsiue IL. Peripheral vs central squamous cell carcinoma of the lung. A comparison of clinical features, histopathology, and survival. Archives of Pathology & Laboratory Medicine 1990;114:468-74.
17. Davidson MR, Gazdar AF, Clarke BE. The pivotal role of pathology in the management of lung cancer. Journal of Thoracic Disease 2013:S463-S78.
18. Travis WD, Brambilla E, Burke A, Marx A, Nicholson AG. WHO classification of tumours of the lung, pleura, thymus and heart. International Agency for Research on Cancer, Lyon; 2015
19. Lortet-Tieulent J, Soerjomataram I, Ferlay J, Rutherford M, Weiderpass E, Bray F. International trends in lung cancer incidence by histological subtype: Adenocarcinoma stabilizing in men but still increasing in women. Lung Cancer 2014;84:13-22.
20. Gabrielson E. Worldwide trends in lung cancer pathology. Respirology (Carlton, Vic) 2006;11:533-8.
21. Gray N. The consequences of the unregulated cigarette. Tobacco Control 2006;15:405-8.
22. Johnson DH, Schiller JH, Bunn PA. Recent Clinical Advances in Lung Cancer Management. Journal of Clinical Oncology 2014;32:973-82.

23. Scagliotti G, Hanna N, Fossella F, et al. The differential efficacy of pemetrexed according to NSCLC histology: a review of two phase III studies. The Oncologist 2009;14:253-63.
24. Socinski MA, Evans T, Gettinger S, et al. Treatment of stage iv non-small cell lung cancer: Diagnosis and management of lung cancer, 3rd ed: american college of chest physicians evidence-based clinical practice guidelines. Chest 2013;143:e341S-e68S.
25. Santos E, Martin-Zanca D, Reddy EP, Pierotti MA, Della Porta G, Barbacid M. Malignant activation of a K-ras oncogene in lung carcinoma but not in normal tissue of the same patient. Science 1984;223:661-4.
26. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497-500.
27. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non–small-cell lung cancer to gefitinib. New England Journal of Medicine 2004;350:2129-39.
28. The Cancer Genome Atlas Research Network. Comprehensive molecular profiling of lung adenocarcinoma. Nature 2014;511:543-50.
29. The Cancer Genome Atlas Research Network. Comprehensive genomic characterization of squamous cell lung cancers. Nature 2012;489:519-25.
30. Chen Z, Fillmore CM, Hammerman PS, Kim CF, Wong K-K. Non-small-cell lung cancers: a heterogeneous set of diseases. Nature Reviews Cancer 2014;14:535-46.
31. The National Lung Screening Trial Research Team. Reduced lung-cancer mortality with low-dose computed tomographic screening. New England Journal of Medicine 2011;365:395-409.
32. Moyer VA. Screening for Lung Cancer: U.S. Preventive services task force recommendation statement. Annals of Internal Medicine 2014;160:330-8.
33. Wender R, Fontham ET, Barrera E, Jr., et al. American Cancer Society lung cancer screening guidelines. CA: a Cancer Journal for Clinicans 2013;63:107-17.
34. Horeweg N, Scholten ET, de Jong PA, et al. Detection of lung cancer through low-dose CT screening (NELSON): a prespecified analysis of screening test performance and interval cancers. The Lancet Oncology;15:1342-50.
35. Helsedirektoratet. Nasjonalt handlingsprogram med retningslinjer for diagnostikk, behandling og oppfølging av lungekreft, mesoteliom og thymom. Helsedirektoratet; 2016.
36. Keith RL, Miller YE. Lung cancer chemoprevention: current status and future prospects. Nature Reviews Clinical Oncology 2013;10:334-43.
37. Heinonen OP, Albanes D. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The New England Journal of Medicine 1994;330:1029-35.
38. Ost DE, Jim Yeung S-C, Tanoue LT, Gould MK. Clinical and organizational factors in the initial evaluation of patients with lung cancer: Diagnosis and management of lung cancer, 3rd ed: american college of chest physicians evidence-based clinical practice guidelines. Chest 2013;143:e121S-e41S.
39. Sobin LH, Gospodarowicz MK, Wittekind C. TNM classification of malignant tumours. Chichester, West Sussex, UK; Hoboken, NJ: Wiley-Blackwell; 2010.
40. Goldstraw P, Crowley J, Chansky K, et al. The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM Classification of malignant tumours. Journal of Thoracic Oncology 2007;2:706-14.
41. Brierley JD, Gospodarowicz MK, Wittekind C. TNM classification of malignant tumours: John Wiley & Sons; 2016.
42. Rami-Porta R, Bolejack V, Giroux DJ, et al. The IASLC lung cancer staging project: the new database to inform the eighth edition of the TNM classification of lung cancer. Journal of Thoracic Oncology 2014;9:1618-24.
43. Goldstraw P, Chansky K, Crowley J, et al. The IASLC lung cancer staging project: proposals for revision of the tnm stage groupings in the forthcoming (eighth) edition of the TNM classification for lung cancer. Journal of Thoracic Oncology 2016;11:39-51.

44. Chang JY, Senan S, Paul MA, et al. Stereotactic ablative radiotherapy versus lobectomy for operable stage I non-small-cell lung cancer: a pooled analysis of two randomised trials. The Lancet Oncology 2015;16:630-7.
45. Louie AV, Palma DA, Dahele M, Rodrigues GB, Senan S. Management of early-stage non-small cell lung cancer using stereotactic ablative radiotherapy: Controversies, insights, and changing horizons. Radiotherapy and Oncology 2015;114:138-47.
46. Kwong KF, Edelman MJ, Suntharalingam M, et al. High-dose radiotherapy in trimodality treatment of Pancoast tumors results in high pathologic complete response rates and excellent long-term survival. The Journal of Thoracic and Cardiovascular Surgery 2005;129:1250-7.
47. Reck M, von Pawel J, Zatloukal P, et al. Overall survival with cisplatin-gemcitabine and bevacizumab or placebo as first-line therapy for nonsquamous non-small-cell lung cancer: results from a randomised phase III trial (AVAiL). Annals of Oncology 2010;21:1804-9.
48. NSCLC Meta-Analyses Collaborative Group. Chemotherapy in addition to supportive care improves survival in advanced non–small-cell lung cancer: a systematic review and meta-analysis of individual patient data from 16 randomized controlled trials. Journal of Clinical Oncology 2008;26:4617-25.
49. Shaw AT, Kim D-W, Mehra R, et al. Ceritinib in alk-rearranged non–small-cell lung cancer. New England Journal of Medicine 2014;370:1189-97.
50. Marrone KA, Brahmer JR. Immune checkpoint therapy in non-small cell lung cancer. Cancer Journal 2016;22:81-91.
51. Pembrolizumab (Keytruda). Indikasjon. (Accessed 20.12, 2016, at https://nyemetoder.no/metoder/pembrolizumab-keytruda-indikasjon-i.)
52. Reck M, Rodríguez-Abreu D, Robinson AG, et al. Pembrolizumab versus chemotherapy for PD-L1–positive non–small-cell lung cancer. New England Journal of Medicine 2016;375:1823-33.
53. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 2012;21:309-22.
54. Bremnes RM, Donnem T, Al-Saad S, et al. The role of tumor stroma in cancer progression and prognosis: emphasis on carcinoma-associated fibroblasts and non-small cell lung cancer. Journal of Thoracic Oncology 2011;6:209-17.
55. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 2011;331:1565-70.
56. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-74.
57. Anagnostou VK, Brahmer JR. Cancer immunotherapy: a future paradigm shift in the treatment of non–small cell lung cancer. Clinical Cancer Research 2015;21:976-84.
58. Virchow RLK. Die krankhaften Geschwülste: A. Hirschwald; 1865.
59. Hagerling C, Casbon A-J, Werb Z. Balancing the innate immune system in tumor development. Trends in Cell Biology;25:214-20.
60. Takahashi H, Ogata H, Nishigaki R, Broide DH, Karin M. Tobacco smoke promotes lung tumorigenesis by triggering IKKβ and JNK1 dependent inflammation. Cancer Cell 2010;17:89.
61. Houghton AM. Mechanistic links between COPD and lung cancer. Nature Reviews Cancer 2013;13:233-45.
62. Kim S, Takahashi H, Lin W-W, et al. Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. Nature 2009;457:102-6.
63. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010;140:883-99.
64. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural innate and adaptive immunity to cancer. Annual Review of Immunology 2011;29:235-71.
65. Koebel CM, Vermi W, Swann JB, et al. Adaptive immunity maintains occult cancer in an equilibrium state. Nature 2007;450:903-7.

66. DuPage M, Cheung A, Mazumdar C, et al. Endogenous T cell responses to antigens expressed in lung adenocarcinomas delay malignant tumor progression. Cancer Cell 2011;19:72-85.
67. DuPage M, Mazumdar C, Schmidt LM, Cheung AF, Jacks T. Expression of tumour-specific antigens underlies cancer immunoediting. Nature 2012;482:405-9.
68. Matsushita H, Vesely MD, Koboldt DC, et al. Cancer exome analysis reveals a t cell dependent mechanism of cancer immunoediting. Nature 2012;482:400-4.
69. Woo SR, Corrales L, Gajewski TF. Innate immune recognition of cancer. Annual Review of Immunology 2015;33:445-74.
70. Motz Greg T, Coukos G. Deciphering and reversing tumor immune suppression. Immunity 2013;39:61-73.
71. Chen Daniel S, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity 2013;39:1-10.
72. De Chaisemartin L, Goc J, Damotte D, et al. Characterization of chemokines and adhesion molecules associated with T cell presence in tertiary lymphoid structures in human lung cancer. Cancer Research 2011;71:6391-9.
73. Brahmer JR, Pardoll DM. Immune checkpoint inhibitors: making immunotherapy a reality for the treatment of lung cancer. Cancer Immunology Research 2013;1:85-91.
74. Mani D, Haigentz M, Aboulafia DM. Lung cancer in hiv infection. Clinical Lung Cancer 2012;13:6-13.
75. Grulich AE, van Leeuwen MT, Falster MO, Vajdic CM. Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. The Lancet;370:59-67.
76. Feng PH, Lee KY, Chang YL, et al. CD14(+)S100A9(+) monocytic myeloid-derived suppressor cells and their clinical relevance in non-small cell lung cancer. American Journal of Respiratory and Critical Care Medicine 2012;186:1025-36.
77. Flavell RA, Sanjabi S, Wrzesinski SH, Licona-Limón P. The polarization of immune cells in the tumour environment by TGFβ. Nature Reviews Immunology 2010;10:554-67.
78. Ganesan AP, Johansson M, Ruffell B, et al. Tumor-infiltrating regulatory T cells inhibit endogenous cytotoxic T cell responses to lung adenocarcinoma. Journal of Immunology 2013;191:2009-17.
79. Granville CA, Memmott RM, Balogh A, et al. A central role for FOXP3+ regulatory T cells in k-ras-driven lung tumorigenesis. PloS one 2009;4:e5061.
80. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nature Reviews Cancer 2012;12:252-64.
81. Baumeister SH, Freeman GJ, Dranoff G, Sharpe AH. Coinhibitory pathways in immunotherapy for cancer. Annual Review of Immunology 2016;34:539-73.
82. Anderson Ana C, Joller N, Kuchroo Vijay K. Lag-3, Tim-3, and TIGIT: Co-inhibitory receptors with specialized functions in immune regulation. Immunity 2016;44:989-1004.
83. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. Science 1996;271:1734-6.
84. van Elsas A, Hurwitz AA, Allison JP. Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. The Journal of Experimental Medicine 1999;190:355-66.
85. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. New England Journal of Medicine 2010;363:711-23.
86. Brahmer JR, Tykodi SS, Chow LQM, et al. Safety and activity of anti–PD-L1 antibody in patients with advanced cancer. The New England Journal of Medicine 2012;366:2455-65.
87. Topalian SL, Hodi FS, Brahmer JR, et al. safety, activity, and immune correlates of anti–PD-1 antibody in cancer. New England Journal of Medicine 2012;366:2443-54.

88. Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. Annual Review of Immunology 2014;32:659-702.
89. Raman D, Sobolik-Delmaire T, Richmond A. Chemokines in health and disease. Experimental Cell Research 2011;317:575-89.
90. Chow MT, Luster AD. Chemokines in cancer. Cancer Immunology Research 2014;2:1125-31.
91. Wendel M, Galani IE, Suri-Payer E, Cerwenka A. Natural killer cell accumulation in tumors is dependent on IFN-gamma and CXCR3 ligands. Cancer Research 2008;68:8437-45.
92. Andersson A, Yang SC, Huang M, et al. IL-7 promotes CXCR3 ligand-dependent T cell antitumor reactivity in lung cancer. Journal of Immunology 2009;182:6951-8.
93. Balkwill FR. The chemokine system and cancer. The Journal of Pathology 2012;226:148-57.
94. Kumar V, Patel S, Tcyganov E, Gabrilovich DI. The nature of myeloid-derived suppressor cells in the tumor microenvironment. Trends in Immunology 2016;37:208-20.
95. Qian B-Z, Li J, Zhang H, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature 2011;475:222-5.
96. Rivas-Fuentes S, Salgado-Aguayo A, Pertuz Belloso S, Gorocica Rosete P, Alvarado-Vasquez N, Aquino-Jarquin G. Role of chemokines in non-small cell lung cancer: angiogenesis and inflammation. Journal of Cancer 2015;6:938-52.
97. Zhang C, Li J, Han Y, Jiang J. A meta-analysis for CXCR4 as a prognostic marker and potential drug target in non-small cell lung cancer. Journal of Drug Design Development and Therapy 2015;9:3267-78.
98. Wald O, Shapira OM, Izhar U. CXCR4/CXCL12 axis in non small cell lung cancer (NSCLC) pathologic roles and therapeutic potential. Theranostics 2013;3:26-33.
99. Bremnes RM, Al-Shibli K, Donnem T, et al. The role of tumor-infiltrating immune cells and chronic inflammation at the tumor site on cancer development, progression, and prognosis: emphasis on non-small cell lung cancer. Journal of Thoracic Oncology 2011;6:824-33.
100. Bremnes RM, Busund LT, Kilvaer TL, et al. The role of tumor-infiltrating lymphocytes in development, progression, and prognosis of non-small cell lung cancer. Journal of Thoracic Oncology.
101. Galon J, Fridman WH, Pages F. The adaptive immunologic microenvironment in colorectal cancer: a novel perspective. Cancer Research 2007;67:1883-6.
102. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nature Reviews Cancer 2012;12:298-306.
103. Dieu-Nosjean M-C, Antoine M, Danel C, et al. Long-term survival for patients with non–small-cell lung cancer with intratumoral lymphoid structures. Journal of Clinical Oncology 2008;26:4410-7.
104. Geng Y, Shao Y, He W, et al. Prognostic role of tumor-infiltrating lymphocytes in lung cancer: a meta-analysis. Cellular Physiology and Biochemistry 2015;37:1560-71.
105. Goldstraw P, Ball D, Jett JR, et al. Non-small-cell lung cancer. The Lancet 2011;378:1727-40.
106. Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006;313:1960-4.
107. Rozek LS, Schmit SL, Greenson JK, et al. Tumor-infiltrating lymphocytes, crohn's-like lymphoid reaction, and survival from colorectal cancer. Journal of the National Cancer Institute 2016;108.
108. Kirilovsky A, Marliot F, El Sissy C, Haicheur N, Galon J, Pagès F. Rational bases for the use of the Immunoscore in routine clinical settings as a prognostic and predictive biomarker in cancer patients. International Immunology 2016;28:373-82.
109. Hermitte F. Biomarkers immune monitoring technology primer: Immunoscore® Colon. Journal for Immunotherapy of Cancer 2016;4:57.
110. Rami-Porta R, Asamura H, Brierley J, Goldstraw P. Staging, tumor profile, and prognostic groups in lung cancer or the new tower of Babel. Journal of Thoracic Oncology 2016;11:1201-3.

111. Donnem T, Kilvaer TK, Andersen S, et al. Strategies for clinical implementation of TNM-Immunoscore in resected nonsmall-cell lung cancer. Annals of Oncology 2015;27:225-32.
112. Burdett S, Rydzewska L, Tierney J, et al. Postoperative radiotherapy for non-small cell lung cancer. Cochrane Database of Systematic Reviews 2016.
113. Corso CD, Rutter CE, Wilson LD, Kim AW, Decker RH, Husain ZA. Re-evaluation of the role of postoperative radiotherapy and the impact of radiation dose for non–small-cell lung cancer using the national cancer database. Journal of Thoracic Oncology 2015;10:148-55.
114. Wang EH, Corso CD, Rutter CE, et al. Postoperative radiation therapy is associated with improved overall survival in incompletely resected stage II and iii non–small-cell lung cancer. Journal of Clinical Oncology 2015;33:2727-34.
115. Formenti SC, Demaria S. Combining Radiotherapy and Cancer Immunotherapy: A paradigm shift. Journal of the National Cancer Institute 2013;105:256-65.
116. Al-Shibli KI, Donnem T, Al-Saad S, Persson M, Bremnes RM, Busund LT. Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer. Clinical Cancer Research 2008;14:5220-7.
117. Al-Shibli K, Al-Saad S, Donnem T, Persson M, Bremnes RM, Busund L-T. The prognostic value of intraepithelial and stromal innate immune system cells in non-small cell lung carcinoma. Histopathology 2009;55:301-12.
118. Matloubian M, David A, Engel S, Ryan JE, Cyster JG. A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. Nature Immunology 2000;1:298-304.
119. Shimaoka T, Nakayama T, Fukumoto N, et al. Cell surface-anchored SR-PSOX/CXC chemokine ligand 16 mediates firm adhesion of CXC chemokine receptor 6-expressing cells. Journal of Leukocyte Biology 2004;75:267-74.
120. Shimaoka T, Kume N, Minami M, et al. Molecular cloning of a novel scavenger receptor for oxidized low density lipoprotein, SR-PSOX, on macrophages. Journal of Biological Chemistry 2000;275:40663-6.
121. Gao Q, Zhao YJ, Wang XY, et al. CXCR6 upregulation contributes to a proinflammatory tumor microenvironment that drives metastasis and poor patient outcomes in hepatocellular carcinoma. Cancer Research 2012;72:3546-56.
122. Wang J, Lu Y, Wang J, Koch AE, Zhang J, Taichman RS. CXCR6 induces prostate cancer progression by the AKT/mammalian target of rapamycin signaling pathway. Cancer Research 2008;68:10367-76.
123. Hojo S, Koizumi K, Tsuneyama K, et al. High-level expression of chemokine CXCL16 by tumor cells correlates with a good prognosis and increased tumor-infiltrating lymphocytes in colorectal cancer. Cancer Research 2007;67:4725-31.
124. Gutwein P, Schramme A, Sinke N, et al. Tumoural CXCL16 expression is a novel prognostic marker of longer survival times in renal cell cancer patients. European Journal of Cancer 2009;45:478-89.
125. Harty JT, Tvinnereim AR, White DW. CD8+ T cell effector mechanisms in resistance to infection. Annual Review of Immunology 2000;18:275-308.
126. Apetoh L, Smyth MJ, Drake CG, et al. Consensus nomenclature for CD8(+) T cell phenotypes in cancer. Oncoimmunology 2015;4:e998538.
127. Kalos M, June Carl H. Adoptive T Cell Transfer for Cancer Immunotherapy in the Era of Synthetic Biology. Immunity 2013;39:49-60.
128. Okazaki T, Okazaki I-m, Wang J, et al. PD-1 and LAG-3 inhibitory co-receptors act synergistically to prevent autoimmunity in mice. The Journal of Experimental Medicine 2011;208:395-407.
129. Andrews LP, Marciscano AE, Drake CG, Vignali DAA. LAG3 (CD223) as a cancer immunotherapy target. Immunological Reviews 2017;276:80-96.
130. He Y, Yu H, Rozeboom L, et al. LAG-3 protein expression in non-small cell lung cancer and its relationship with PD-1/PD-L1 and tumor-infiltrating lymphocytes. Journal of Thoracic Oncology 2017. DOI: 10.1016/j.jtho.2017.01.019

131. Battifora H. The multitumor (sausage) tissue block: novel method for immunohistochemical antibody testing. Laboratory Investigation. 1986;55:244-8.
132. Battifora H, Mehta P. The checkerboard tissue block. An improved multitissue control block. Laboratory investigation 1990;63:722-4.
133. Kononen J, Bubendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nature Medicine 1998;4:844-7.
134. Simon R. Applications of tissue microarray technology. Methods in Molecular Biology 2010;664:1-16.
135. Camp RL, Neumeister V, Rimm DL. A decade of tissue microarrays: Progress in the discovery and validation of cancer biomarkers. Journal of Clinical Oncology 2008;26:5630-7.
136. O'Hurley G, Sjöstedt E, Rahman A, et al. Garbage in, garbage out: A critical evaluation of strategies used for validation of immunohistochemical biomarkers. Molecular Oncology 2014;8:783-98.
137. Coons AH, Creech HJ, Jones RN. Immunological properties of an antibody containing a fluorescent group. Experimental Biology and Medicine 1941;47:200-2.
138. de Matos LL, Trufelli DC, de Matos MGL, da Silva Pinhal MA. Immunohistochemistry as an I mportant Tool in Biomarkers Detection and Clinical Practice. Biomarker Insights 2010;5:9-20.
139. Lipman NS, Jackson LR, Trudel LJ, Weis-Garcia F. Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. ILAR Journal 2005;46:258-68.
140. Travis WD, Brambilla E, Müller-Hermelink HK, Harris CCE. World Health Organization classification of tumours. Pathology & genetics, tumours of the lung, pleura, thymus and heart. Lyon, France: International Agency for Research on Cancer; 2004.
141. Travis WD, Brambilla E, Noguchi M, et al. International association for the study of lung cancer/american thoracic society/european respiratory society international multidisciplinary classification of lung adenocarcinoma. Journal of Thoracic Oncology 2011;6:244-85.
142. Pohl M, Olsen KE, Holst R, Ditzel HJ, Hansen O. Tissue microarrays in non-small-cell lung cancer: reliability of immunohistochemically-determined biomarkers. Clinical Lung Cancer 2014;15:222-30.e3.
143. Rud AK, Lund-Iversen M, Berge G, et al. Expression of S100A4, ephrin-A1 and osteopontin in non-small cell lung cancer. BMC Cancer 2012;12:333-.
144. A Package for Survival Analysis in S_. version 2.38. 2015. at https://CRAN.R-project.org/package=survival.)
145. Wickham H. ggplot2: elegant graphics for data analysis. Springer New York 2009.
146. survminer: Drawing Survival Curves using 'ggplot2. 2016. at https://CRAN.R-project.org/package=survminer.)
147. Dønnem T. Molecular analysis of angiogenic markers as prognostic factors for non-small cell lung cancer (NSCLC): University of Tromsø; 2009.
148. Dorsett Y, Tuschl T. siRNAs: applications in functional genomics and potential as therapeutics. Nat Rev Drug Discov 2004;3:318-29.
149. Leighl NB. Positron Emission Tomography use and stage migration in lung cancer: a false s ense of accomplishment? Journal of Clinical Oncology 2012;30:2710-1.
150. de Bruin EC, McGranahan N, Mitter R, et al. Spatial and temporal diversity in genomic instability processes defines lung cancer evolution. Science 2014;346:251-6.
151. Sauter G. Representativity of TMA studies. Methods in Molecular Biology 2010;664:27-35.
152. Nocito A, Bubendorf L, Tinner EM, et al. Microarrays of bladder cancer tissue are highly representative of proliferation index and histological grade. The Journal of Pathology 2001;194:349-57.
153. Garcia JF, Camacho FI, Morente M, et al. Hodgkin and Reed-Sternberg cells harbor alterations in the major tumor suppressor pathways and cell-cycle checkpoints: analyses using tissue microarrays. Blood 2003;101:681-9.

154. Schmidt LH, Biesterfeld S, Kummel A, et al. Tissue microarrays are reliable tools for the clinicopathological characterization of lung cancer tissue. Anticancer Research 2009;29:201-9.
155. Leversha MA, Fielding P, Watson S, Gosney JR, Field JK. Expression of p53, pRB, and p16 in lung tumours: a validation study on tissue microarrays. The Journal of Pathology 2003;200:610-9.
156. Choi PJ, Yang DK, Son CH, Lee KE, Lee JI, Roh MS. Fascin immunoreactivity for preoperatively predicting lymph node metastases in peripheral adenocarcinoma of the lung 3 cm or less in diameter. European Journal of Cardio-Thoracic Surgery 2006;30:538-42.
157. Xie R, Chung J-Y, Ylaya K, et al. Factors influencing the degradation of archival formalin-fixed paraffin-embedded tissue sections. Journal of Histochemistry and Cytochemistry 2011;59:356-65.
158. Litlekalsoy J, Vatne V, Hostmark JG, Laerum OD. Immunohistochemical markers in urinary bladder carcinomas from paraffin-embedded archival tissue after storage for 5–70 years. BJU International 2007;99:1013-9.
159. Chetcuti A, Mackie N, Tafavogh S, et al. Can archival tissue reveal answers to modern research questions?: computer-aided histological assessment of neuroblastoma tumours collected over 60 years. Microarrays 2014;3:72-88.
160. Seidal T, Balaton AJ, Battifora H. Interpretation and quantification of immunostains. The American Journal of Surgical Pathology 2001;25:1204-7.
161. Donnem T, Al-Saad S, Al-Shibli K, et al. Inverse prognostic impact of angiogenic marker expression in tumor cells versus stromal cells in non small cell lung cancer. Clinical Cancer Research 2007;13:6649-57.
162. Altman DG, Lausen B, Sauerbrei W, Schumacher M. Dangers of using "optimal" cutpoints in the evaluation of prognostic factors. Journal of the National Cancer Institute 1994;86:829-35.
163. Hiraoka K, Miyamoto M, Cho Y, et al. Concurrent infiltration by CD8+ T cells and CD4+ T cells is a favourable prognostic factor in non-small-cell lung carcinoma. British Journal of Cancer 2006;94:275-80.
164. Balermpas P, Michel Y, Wagenblast J, et al. Tumour-infiltrating lymphocytes predict response to definitive chemoradiotherapy in head and neck cancer. British Journal of Cancer 2014;110:501-9.
165. Lee Y, Auh SL, Wang Y, et al. Therapeutic effects of ablative radiation on local tumor require CD8+ T cells: changing strategies for cancer treatment. Blood 2009;114:589-95.
166. Golden EB, Frances D, Pellicciotta I, Demaria S, Helen Barcellos-Hoff M, Formenti SC. Radiation fosters dose-dependent and chemotherapy-induced immunogenic cell death. Oncoimmunology 2014;3:e28518.
167. Twyman-Saint Victor C, Rech AJ, Maity A, et al. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. Nature 2015;520:373-7.
168. Kalbasi A, Komar C, Tooker GM, et al. Tumor-Derived CCL2 Mediates Resistance to Radiotherapy in Pancreatic Ductal Adenocarcinoma. Clinical Cancer Research 2017;23:137-48.
169. Medler TR, Cotechini T, Coussens LM. Immune response to cancer therapy: mounting an effective antitumor response and mechanisms of resistance. Trends in cancer 2015;1:66-75.
170. Hanna GG, Illidge T. Radiotherapy and Immunotherapy Combinations in Non-small Cell Lung Cancer: A Promising Future? Clinical Oncology 2016;28:726-31.
171. Brambilla E, Le Teuff G, Marguet S, et al. Prognostic effect of tumor lymphocytic infiltration in resectable non–small-cell lung cancer. Journal of Clinical Oncology 2016;34:1223-30.
172. Falkson CB, Vella ET, Yu E, et al. Radiotherapy with curative intent in patients with early-stage, medically inoperable, non-small-cell lung cancer: A Systematic Review. Clinical Lung Cancer 2016.

173. Kalbasi A, Beatty GL, Berman AT. Expanding tumor lymphocytic infiltration as a prognostic tool to patients with nsclc who are treated with radiotherapy? Journal of Thoracic Oncology 2016;11:e141-e2.
174. Xing Y-n, Xu X-y, Nie X-c, et al. Role and clinicopathologic significance of CXC chemokine ligand 16 and chemokine (C-X-C motif) receptor 6 expression in gastric carcinomas. Human Pathology 2012;43:2299-307.
175. Cullen R, Germanov E, Shimaoka T, Johnston B. Enhanced tumor metastasis in response to blockade of the chemokine receptor CXCR6 is overcome by NKT cell activation. The Journal of Immunology 2009;183:5807-15.
176. Richardsen E, Ness N, Melbø-Jørgensen C, et al. The prognostic significance of CXCL16 and its receptor C-X-C Chemokine Receptor 6 in prostate cancer. The American Journal of Pathology 2015;185:2722-30.
177. Hu W, Zhen X, Xiong B, Wang B, Zhang W, Zhou W. CXCR6 is expressed in human prostate cancer in vivo and is involved in the in vitro invasion of PC3 and LNCap cells. Cancer Science 2008;99:1362-9.
178. Hu W, Liu Y, Zhou W, Si L, Ren L. CXCL16 and CXCR6 are coexpressed in human lung cancer in vivo and mediate the invasion of lung cancer cell lines in vitro. PloS one 2014;9:e99056.
179. Mir H, Singh R, Kloecker GH, Lillard Jr. JW, Singh S. CXCR6 expression in non-small cell lung carcinoma supports metastatic process via modulating metalloproteinases. Oncotarget 2015;6.
180. Gooden MJ, Wiersma VR, Boerma A, et al. Elevated serum CXCL16 is an independent predictor of poor survival in ovarian cancer and may reflect pro-metastatic ADAM protease activity. Br J Cancer 2014;110:1535-44.
181. Obeid JM, Wages NA, Hu Y, Deacon DH, Slingluff CL. Heterogeneity of CD8+ tumor-infiltrating lymphocytes in non-small-cell lung cancer: impact on patient prognostic assessments and comparison of quantification by different sampling strategies. Cancer Immunology, Immunotherapy 2017;66:33-43.
182. Schalper KA, Brown J, Carvajal-Hausdorf D, et al. Objective measurement and clinical significance of TILs in non-small cell lung cancer. Jourral of the National Cancer Institute 2015;107.
183. Giraldo NA, Becht E, Pagès F, et al. Orchestration and Prognostic Significance of Immune Checkpoints in the Microenvironment of Primary and Metastatic Renal Cell Cancer. Clinical Cancer Research 2015;21:3031-40.
184. Deng WW, Mao L, Yu GT, et al. LAG-3 confers poor prognosis and its blockade reshapes antitumor response in head and neck squamous cell carcinoma. Oncoimmunology 2016;5:e1239005.
185. Kim HR, Ha S-J, Hong MH, et al. PD-L1 expression on immune cells, but not on tumor cells, is a favorable prognostic factor for head and neck cancer patients. Scientific Reports 2016;6:36956.
186. Tassi E, Grazia G, Vegetti C, et al. Early effector T lymphocytes coexpress multiple inhibitory receptors in primary non-small cell lung cancer. Cancer Research 2016. DOI: 10.1158/0008-5472.can-16-1387
187. Gros A, Robbins PF, Yao X, et al. PD-1 identifies the patient-specific CD8(+) tumor-reactive repertoire infiltrating human tumors. The Journal of Clinical Investigation 2014;124:2246-59.