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The prevalence and prognostic significance of endocrinology-related biomarkers in non-small cell lung cancer

A retrospective study evaluating biomarkers in lung tumor microenvironment by immunohistochemistry and in situ hybridization on tissue microarrays

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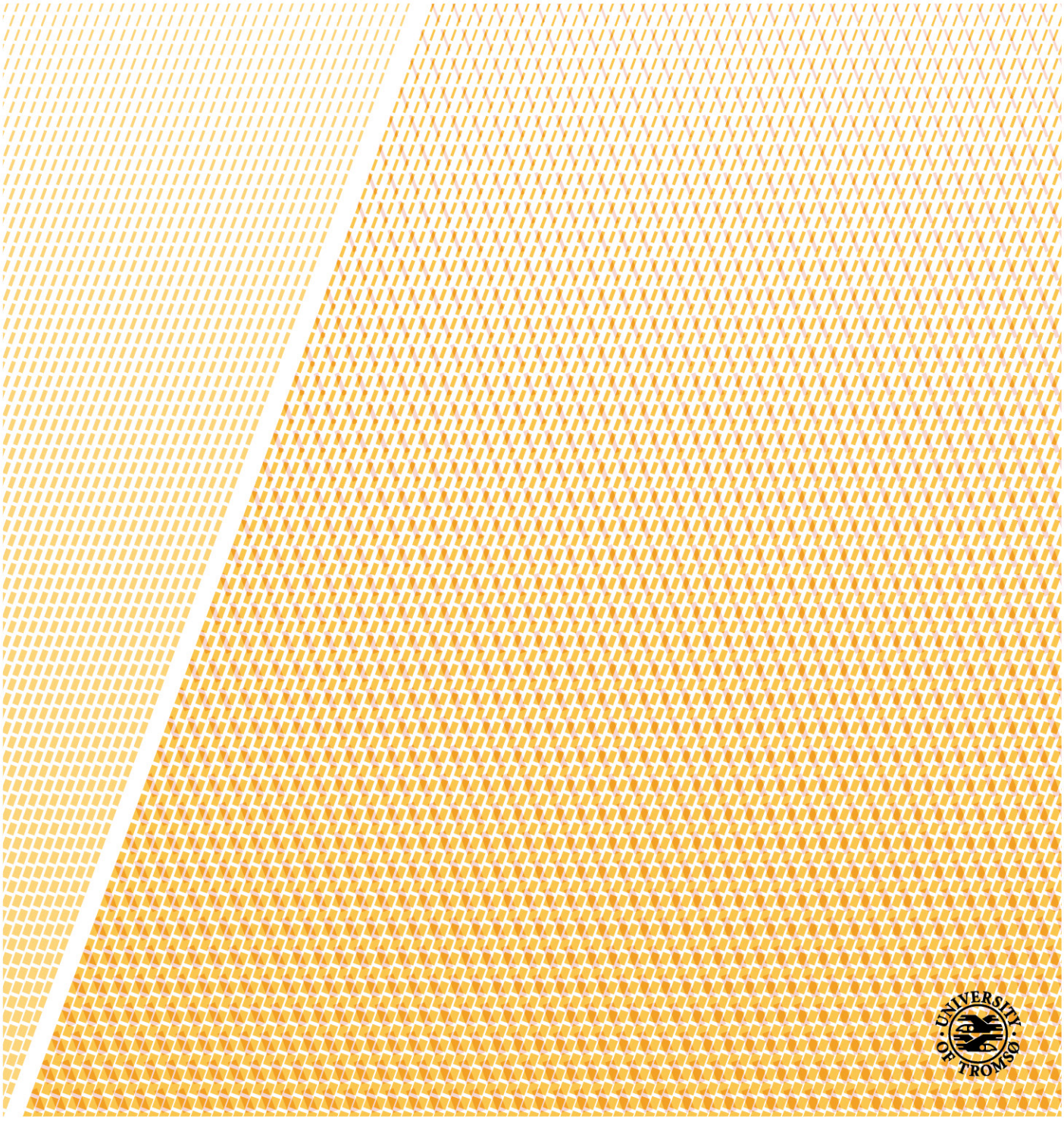
A dissertation for the degree of Philosophiae Doctor



“En god resept på et godt liv, er å gi litt mer faen”

Per Fugelli

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LIST OF PAPERS

The thesis is based on the following papers:

PAPER I

Skjefstad K, Richardsen E, Donnem T, Andersen S, Kiselev Y, Grindstad T, Hald S, Bremnes RM, Busund LT, Al-Saad S. The prognostic role of progesterone receptor expression in non-small cell lung cancer patients: Gender-related impacts and correlation with disease-specific survival. Steroids. 2015 Jun;98:29-36.

PAPER II

Skjefstad K, Grindstad T, Khanehkenari MR, Richardsen E, Donnem T, Kilvaer T, Andersen S, Bremnes RM, Busund LT, Al-Saad S. Prognostic relevance of estrogen receptor α, β and aromatase expression in non-small cell lung cancer. Steroids. 2016 Sep;113:5-13.

PAPER III

Skjefstad K, Johannessen C, Grindstad T, Kilvaer T, Paulsen EE, Pedersen M, Donnem T, Andersen S, Bremnes R, Richardsen E, Al-Saad S, Busund LT. A gender specific improved survival related to stromal miR-143 and miR-145 expression in non-small cell lung cancer. (*Submitted*)

ABBREVIATIONS

AAH	Atypical adenomatous hyperplasia
Ab	Antibody
ADC	Adenocarcinoma
AIS	Adenocarcinoma *in situ*
ALK	Anaplastic lymphoma kinase
AR	Aromatase enzyme
CAFs	Carcinoma-associated fibroblasts
CT	Computed tomography
DSS	Disease-specific survival
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
HER-2	Human epidermal growth factor receptor 2
HR	Hazard ratio
IASCLC	International Association for the Study of Lung Cancer
IHC	Immunohistochemistry
IGF-1	Insulin-like growth factor 1
IGFR-1	Insulin-like growth factor receptor 1
ISH	*In situ* hybridization
LCC	Large cell carcinoma
MAb	Monoclonal antibody

miRNA	microRNA
MMP	Matrix metalloproteinase
N+	Metastatic lymph node
NSCLC	Non-small cell lung cancer
P	P-value
PAb	Polyclonal antibody
PDGFR	Platelet-derived growth factor receptor
PET	Positron emission tomography
PR	Progesterone receptor
PT	Primary tumor
RNA	Ribonucleic acid
RT	Radiation therapy
RT-PCR	Real-time polymerase chain reaction
SCC	Squamous cell carcinoma
SCLC	Small cell carcinoma
SHR	Sex steroid hormone receptor
TGF-β	Transforming growth factor β
TME	Tumor microenvironment
TNM	Tumor, node, metastasis
TIS	Tumor *in situ*
UICC	Union for International Cancer Control
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

INTRODUCTION

Despite the progress in the field of cancer research and clinical management, the 5-year survival in lung cancer remains dramatically poor. Annually, lung cancer kills more people worldwide than breast cancer, colorectal cancer and prostate cancer combined [1]. Nevertheless, lung cancer research receives less funding than either of the aforementioned cancer types, both globally and nationally[2,3].

Early diagnostics and treatment have improved lung cancer survival, however the majority of patients will relapse and die within few years after initial diagnosis. Poor and diverse treatment response in and between patients represents a major challenge, and the need for an improved and more personalized treatment strategy is crucial.

Tobacco smoking is the major contributor to this disease, yet a number of never-smokers, especially women, are developing lung cancer today. This emphasizes the need to explore additional risk factors and causes of lung cancer.

Our group previously revealed a gender related significance of human epidermal growth factor receptor 2 (HER-2) in NSCLC, confirming reports indicating gender-related differences in NSCLC[4,5]. To further elucidate the potential of a gender-related subtyping of NSCLC patients, we herein investigated the prevalence and prognostic role of sex steroid hormone receptors (SHRs) in NSCLC. By constructing tissue microarrays from cohorts of surgically resected NSCLC patients, we analyzed the expression and prognostic impact of the SHRs progesterone receptor (PR), estrogen receptor α (ERα) and β (ERβ), and the aromatase enzyme (AR). Further, we analyzed the prevalence and the prognostic role of the microRNA cluster miR-143/miR-145, reported to be associated with the estrogenic pathway in malignant growth. Our results confirm gender-related molecular discrepancies in NSCLC, indicating a potential role of endocrinology-related biomarkers in NSCLC patients.

1 BACKGROUND

1.1 Lung cancer

1.1.1 Epidemiology

Today, lung cancer has become the deadliest cancer type worldwide, and accounts for 1.6 million lung cancer deaths, each year[1]. Geographical variations exists, and the age-adjusted incidence rates are highest in Central and Eastern Europe for males (43.5 in 100.000) and Northern America (33.8 in 100.000) for females[6]. Mortality rates follow the incidence rates with regards to geographical patterns[1]. In Norway, 1615 males and 1465 females were diagnosed with lung cancer in 2016. This represents a 6% decrease for male incidence, but a fearsome 9% increase in female incidence in the most recent five-year period, compared to the previous five-year period (**Figure 1**)[7]. Historically speaking, the large majority of lung cancer patients used to be male. In Norway, the gender difference has evened out during the last decades with a male:female ratio of 1.3 today compared to 4.0 in 1982[7].

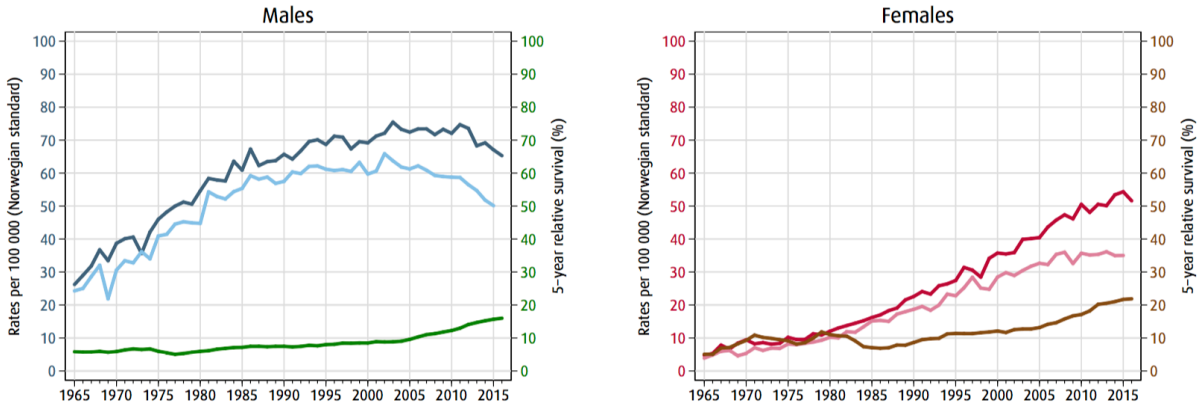

Figure 1 *Lung cancer incidence (top line) and mortality (middle line) rates from 1965-2016 in Norway. 5-year survival (bottom line) is presented on the right side of the curve. Figure adapted from "Cancer incidence, mortality, survival and prevalence in Norway, Cancer in Norway 2016"[7].*

Globally, however, gender rates vary greatly with some countries reporting an increasing male and female incidence (Brazil, Japan), while others are reporting a declining incidence in both genders (Hong Kong, the U.S.). Observations from Canada and Denmark show a decrease in male incidence accompanied by an increase in female incidence, as is observed in Norway (**Figure 1**)[6].

1.1.2 Etiology and risk factors

Cigarette smoking remains the predominant risk factor for developing lung cancer and accounts for 80-90% of lung cancer cases in the Nordic region[8]. Prior to the smoking epidemic reaching its peak in the 1950's, lung cancer was a rare disease[9]. There is a steep increase in relative risk of developing lung cancer according to smoking duration and number of cigarettes smoked per day[10]. The risk seems to be similar for both men and women, given the same level of tobacco consumption[11-13]. Additionally, genomic mapping has revealed a 10-fold higher gene mutation prevalence in smokers, compared to non-smokers, further increasing the chance of neoplastic development[14]. Other risk factors include passive smoking, previous lung disease, radon exposure, air pollution, genetic susceptibility and occupational pollutions including asbestos, arsenic and silica[14-16]. There has been a significant reduction in every-day smokers in the last 20 years, with only 15% of the Norwegian population reporting daily tobacco smoking in 2014[8]. Lung cancer deaths are declining today, reflecting the decrease primarily in the number of male smokers. For females, there has been an increase in the annual lung cancer incidence, most likely due to the fact that historically, women started smoking later than men, but the lung cancer death rate seems to have reached a plateau[17]. The majority of never-smokers with lung cancer are women and an increase in lung cancer incidence among never-smoking females is observed, rendering the possibility of another important risk factor contributing to the disease, besides smoking[10]. Female NSCLC patients live longer than males suffering from the same disease, and older women (>60 years)

show improved survival compared to younger women with the same disease[8,18,19]. This may suggest gender-related differences in the pathogenesis of lung cancer, and a possible role for sex steroid hormones as pre-menopausal women are subjected to higher levels of circulating sex hormones than post-menopausal women. Several large, randomized studies have reported associations between estrogen plus progestin therapy and increased lung cancer incidence and mortality in females, further proposing sex steroids as contributors to this disease[20-23].

Age is a risk factor for developing lung cancer in both smokers and non-smokers with a median age at diagnosis close to 70 years, and represents a negative prognosticator for lung cancer survival (**Figure 2**)[7,24,25].

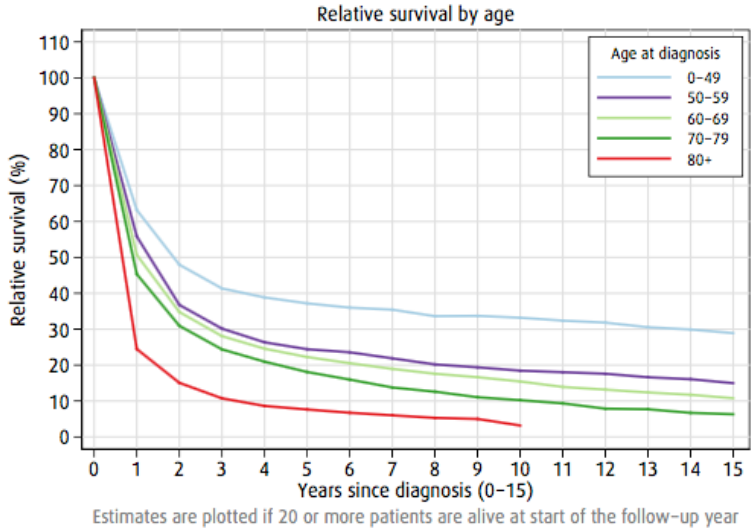

Figure 2 *Trends in survival according to age at diagnosis. Figure adapted from "Cancer incidence, mortality, survival and prevalence in Norway, Cancer in Norway 2016"*[7].

The risk of developing lung cancer increases in patients with chronic disease and dysregulated immune response (e.g. HIV), which both are described as contributors to the development of neoplastic disease[26].

1.1.3 Histopathology

1.1.3.1 Premalignant lesions of the lung

The histological components constituting the respiratory tree may be divided into central and peripheral compartments, serving different physiological roles in respiration[27]. Chronic irritation of central airways (e.g. tobacco smoking, air pollution) may cause aberrant maturation and differentiation of normal bronchial epithelium into squamous cell metaplasia[27]. Subsequently, squamous dysplasia may occur in various degrees. A severe degree of dysplasia, designated as carcinoma *in situ*, may progress to an infiltrating malignant tumor[28]. Atypical adenomatous hyperplasia (AAH) and adenocarcinoma *in situ* (AIS) may arise in pneumocytes or bronchiolar secretory cells, and is often localized more peripherally[29]. The accumulation of molecular alterations causing the development of premalignant lesions have been intensively explored in order to reveal biomarkers that may aid early detection of lung cancer[28,30].

1.1.3.2 Histopathology of lung cancer

As previously mentioned, a further progression of premalignant lesions of the lung will eventually lead to the development of invasive lung carcinoma, where non-small cell lung cancer (NSCLC) is the predominant type of primary carcinoma of the lung (85% of cases) and small cell lung carcinoma (SCLC) comprises the remaining 15 %[24]. NSCLC is further classified based on morphology into histopathological subtypes and in a subset of cases by additional immunohistochemical (IHC) analysis, as recommended in the 2015 World Health Organization (WHO) Classification[31]. This is in contrast with previous WHO recommendations where light microscopy of hematoxylin and eosin stained tissue samples mainly based on morphology, was recommended in the classification routine. The importance of sub-classifying the major pathological groups in NSCLC in squamous cell carcinoma (SCC) and adenocarcinoma (ADC), has gained new attention parallel with the development of

personalized medicine in lung cancer treatment[32]. There is an increasing need for specific mapping of histologic and genetic characteristics of tumors in order to tailor treatment, as targeted treatment are becoming widely available in the management of NSCLC[33].

Historically, SCC has been the most common subtype of NSCLC, but due to changes in smoking behavior ADC now represents the predominant NSCLC subtype[28]. During the late 50's, filtered cigarettes were introduced allowing deeper inhalation of carcinogens. Further, the diagnostic advancements facilitating biopsies of the small, peripheral tumors often comprised of ADC, increased the ADC frequency[34,35]. ADC comprises the dominant subtype in non-smokers, women and Asian patients. Smoking is however, the most common cause of ADC, as with SCC[28,35]. Histologically, ADCs are characterized by cells forming glandular structures and occasional mucus production, while SCC may be keratinizing, non-keratinizing or showing a lower differentiated basaloid morphology[28].

To accurately sub-classify the histological groups, specific IHC markers are utilized: the thyroid transcription factor (TTF1) is expressed by pneumocytes and in most ADCs. The transformation-related protein 63 (p63) and more specifically its isoform p40 are expressed in bronchial epithelium and in most SCCs[8]. Despite challenges including cross-reaction with normal pulmonary and bronchial cells, IHC staining for these markers is of acceptable sensitivity and specificity[36]. The previously more common subtype undifferentiated large-cell carcinoma (LCC) constitutes now only an exclusion diagnosis (3% of cases), if no IHC markers characterizing SCC or ADC are observed in tumor cells[28]. Targeted therapy is becoming widely available in NSCLC treatment, thus all resected NSCLC tumors are immunohistochemically tested for the expression of programmed cell death protein ligand 1 (PD-L1). Additionally, all non-SCC lung tumors are tested for the presence of driver mutations in epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK)[8].

Due to tremendous advances in genomic mapping during the last decades, several driver mutations have been described to be associated with NSCLC, the most important being EGFR and ALK, as described in chapter 1.1.3.2 and 1.1.6.2. Other gene mutations often occurring in NSCLC include: KRAS, ROS1, PIK3CA, HER-2, BRAF and AKT[8]. These driver mutations may represent potential therapeutic targets in subgroups of patients, but remain to be further evaluated in long term of clinical practice.

Regardless of histological subtype, the most frequent sites of lung cancer metastasis include bone, brain, adrenal glands and liver and are single handedly responsible for the majority (70%) of lung cancer related deaths[37].

1.1.4 Diagnosis and staging

Symptoms and signs of lung cancer may be vague and unspecific, causing a delay in diagnostics. Cough, shortness of breath, and chest/shoulder pain are common symptoms in non-malignant diseases affecting the lung (e.g. pneumonia), but may also indicate an underlying lung malignancy[8]. Other symptoms include hemoptysis, vena cava superior syndrome and stridor, which all indicate the need of rapid medical attention. A study of 2293 consecutive NSCLC patients reported that at the time of diagnosis, 62.1% and 62.0% of the patients had cardiovascular disease and chronic obstructive pulmonary disease (COPD), respectively[38]. The high degree of co-morbidity may further obscure lung cancer symptoms. Early diagnostics are pivotal in order to treat the patient with a curative intent. Unfortunately, the majority of patients present with an advanced disease at the time of diagnosis, severely limiting the possibility of a curative treatment[8].

The diagnostic procedure of lung cancer includes the determination of histological subtype, tumor stage according to the 8th edition of the TNM (tumor, node, metastasis) classification of malignant tumors, published by the Union for International Cancer Control (UICC), and clinical stage (I-IV)[8,39]. The diagnostics are usually initiated with a chest X-ray upon clinical

suspicion, and a follow-up computer tomography (CT) of the chest and upper abdomen (**Figure 3**).

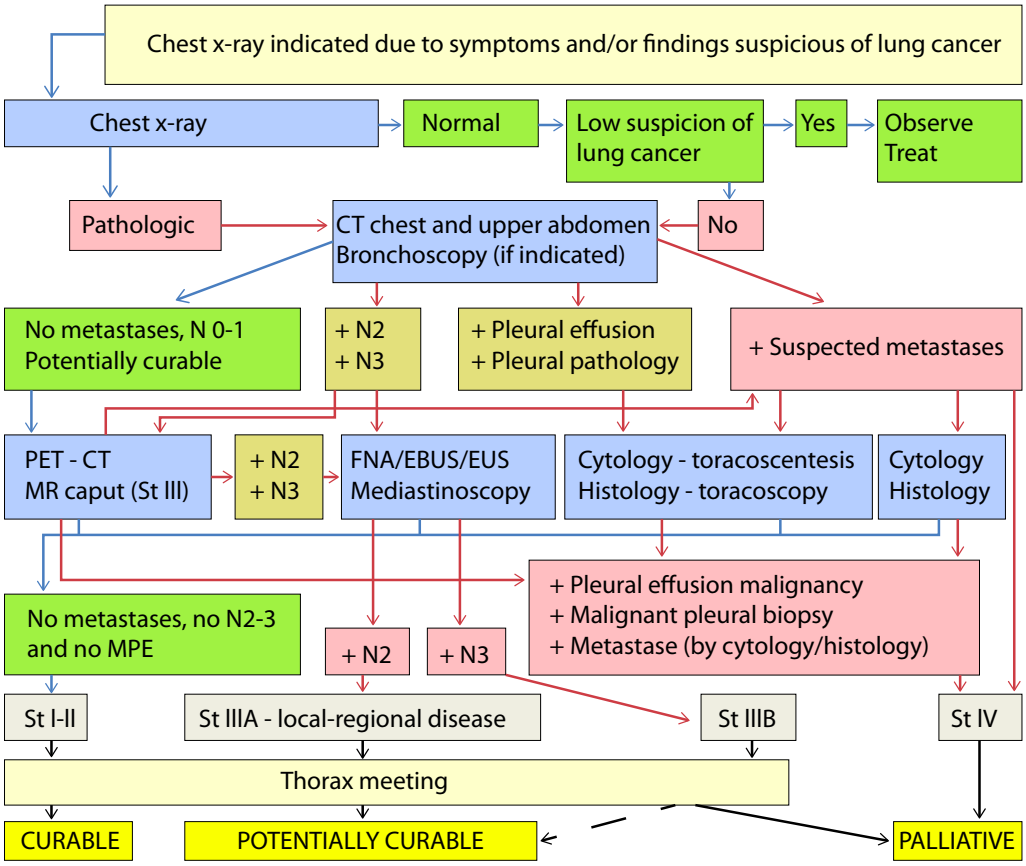

Figure 3 *Adapted from "Nasjonalt handlingsprogram med retningslinjer for diagnostikk, behandling og oppfølging av lungekreft, mesoteliom og thymom"* [8]*, modified by Kaja Skjefstad.*
Abbreviations: PET-CT; positron emission tomography-computed tomography, FNA; fine needle aspiration, EBUS; endobronchial ultrasound, EUS; esophageal ultrasound, MPE; malignant pleural effusion.

Depending on the radiologic results, further diagnostics aim to collect tissue samples for histopathologic evaluation using the method that provides most tumor tissue at the lowest risk for the patient. Further diagnostics are performed according to **Figure 3**. The final decision regarding treatment strategy and whether treatment is according to a curative or palliative aim, is based on multidisciplinary team meetings (radiologist, oncologist, thoracic surgeon,

pulmonary physician and pathologist) and final TNM status[8]. The implementation of the 8th UICC TNM edition started in January 2017 and was based on a database containing 94 708 NSCLC patients from 16 different countries, collected by the International Association for the Study of Lung Cancer (IASCLC) Staging and Prognostic Factors Committee[40]. In the transition from the 7th to the 8th edition, no changes were made in the N descriptors. The 7th UICC TNM edition introduced an enhanced focus on tumor size which was maintained, and further emphasized, in the 8th edition by implementing additional T size cutoff points (**Table 1**)[40,41]. T1N0M0 was sub-divided into T1a, T1b and T1c, according to tumor size. This subsequently caused the implementation of new staging groups: clinical stage IA was further divided into IA1, IA2 or IA3, according to the T descriptor. Further changes included 1) A new stage IIIC: including T3-T4N3M0 cases. This addition was implemented due to a significantly worse prognosis compared to patients with tumors remaining in stage IIIB. 2) Stage IV was subdivided into IVA including any T, any N and M1a or M1b vs. IVB including any T, any N and M1c, 3) Tis (*in situ*) was introduced with recommendations of distinguishing between histologic Tis type (Tis AIS for adenocarcinoma *in situ* and Tis SCIS for squamous cell carcinoma *in situ*)[40,42]. M1c represents a new M descriptor, classifying tumors with multiple metastasis in one or several organs, while M1b represents tumors with a single extra thoracic metastasis[42].

Table 1 *The 8th edition of TNM classification and staging of lung cancer. Adapted from "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"[40].*

Disease Stage	Substage	T descriptor	N descriptor	M descriptor
Occult carcinoma		TX	N0 No regional lymph node metastases	M0 No distant metastases
Stage 0		Tis Carcinoma *in situ*[a]		
Stage I	IA1	T1mi (minimally invasive)[b] T1a Tumor ≤ 1 cm		
	IA2	T1b Tumor > 1 cm ≤ 2 cm		
	IA3	T1c Tumor > 2 cm ≤ 3 cm		
	IB	T2a Tumor > 3 cm ≤ 5 cm[c]		
Stage II	IIA	T2b Tumor > 4 cm ≤ 5 cm[c]		
	IIB	T1a-c	N1[f]	
		T2a-b	N1	
		T3 Tumor > 5 cm ≤ 7 cm[d]	N0	
Stage III	IIIA	T1a-c	N2[g]	
		T2a-b	N2	
		T3	N1	
		T4 Tumor > 7 cm[e]	N0, N1	
	IIIB	T1a-c	N3[h]	
		T2a-b	N3	
		T3	N2	
		T4	N2	
	IIIC	T3	N3	
		T4	N3	
Stage IV	IVA	Any T	Any N	M1a[j]
		Any T	Any N	M1b[k]
	IVB	Any T	Any N	M1c[l]

[a] Tis includes squamous carcinoma *in situ* and adenocarcinoma *in situ*
[b] Solitary adenocarcinoma ≤ 3 cm, with a predominantly lepidic pattern, ≤ 5 mm invasion
[c] T2: tumor < 3 cm, but ≤ 5 cm, or tumor with any of the following characteristics: involves main bronchus, not carina. Invades visceral pleura. Associated with atelectasis or obstructive pneumonitis that extends to the hilar region either involving part of or the entire lung
[d] or any that directly invades: parietal pleura, chest wall (including superior sulcus tumors), phrenic nerve, parietal pericardium; or separate tumor nodule(s) in the same lobe as the primary
[e] or of any size that invades: diaphragm, mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, oesophagus, vertebral body, carina; separate tumor nodule(s) in a different ipsilateral lobe to that of the primary
[f] metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension
[g] metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)
[h] metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)
[j] separate tumor nodule(s) in a contralateral lobe; tumor with pleural or pericardial nodules or malignant pleural or pericardial effusion
[k] single extrathoracic metastasis in a single organ, including a single non-regional node
[l] multiple extrathoracic metastasis in a single or multiple organs

1.1.5 Lung cancer screening

"The principles and practice of screening for disease" was published by the WHO in 1968 to aid the decision process of whether or not to implement a screening program or test for a given disease[43,44]. Herein, 10 key principles are listed including "There should be an agreed upon policy on whom to treat as patients" and "The cost of case-finding should be economically balanced in relation to possible expenditure on medical care as a whole". In addition, documented survival benefit of the screening method is required[8]. The National Lung Screening Trial (NLST), a randomized controlled trial including 53 000 former or current American smokers, reported a 20% reduction in lung cancer mortality rate by the use of low-dose CT for the detection of lung cancer in a high-risk population[45]. Due to these results, several organizations including the National Comprehensive Cancer Network (NCCN) and European Society for Medical Oncology (ESMO) now recommend screening for lung cancer with low-dose CT in selected high-risk populations[39]. Nevertheless, the majority of health institutions internationally await further results and validation prior to implementing lung cancer screening, including Norwegian health authorities. As of today, several of the aforementioned principles listed by the WHO are challenged when evaluating lung cancer screening. A recent publication addresses these challenges upon implementation of lung cancer screening in the Nordic countries, and propose performing CT screening pilot studies prior to implementation[46]. An optimal method confirmed for selection of screening population and a compiled plan for screening organization based on socioeconomic considerations are needed[8]. The cost effectiveness of a lung cancer screening program has only been evaluated in the UK, Canada and the U.S. Detailed calculations should be performed for every screening country prior to implementation of a lung cancer screening trial[46]. The Dutch-Belgian Lung Cancer Screening trial (NELSON), is the second largest trial examining whether low-dose CT screening reduces lung cancer mortality. Results are expected shortly,

and will have great importance in validating, or refuting, the mortality reduction reported from the NLST trial[45,47].

1.1.6 Prognosis and treatment

Established positive prognostic factors in NSCLC patients include early-stage disease at time of diagnosis, female gender, no significant weight loss (< 5% for 3 months or < 10% for 6 months), and a good performance status assessed by the Eastern Cooperative Oncology Group (ECOG) status[39].

For all stages combined the relative 5-year survival in the time span 2012-2016 was 18% in Norway. This represented 22 % for female patients and 16 % for male patients. If surgically treated, the percentages increase to 56% and 41%, respectively[8]. This demonstrates the importance of early disease detection, enabling curative surgical treatment.

Regardless of curative or palliative treatment strategy, smoking cessation is of pivotal importance for all NSCLC patients, as smoking interacts with cancer therapy and is associated with an increased risk of death[39,48]. Current smokers diagnosed with NSCLC should therefore be offered smoking cessation support and counseling.

1.1.6.1 Curative treatment

Stage I and II disease (T1-T2, N0-1) are considered surgically resectable, in addition to a minority of cases with stage IIIA disease (T3, N1)[49]. However, surgery is only performed in patients considered medically and technically suitable for the surgical procedure. Patients considered inoperable or patients not willing to undergo surgical procedures, may be candidates for radical radiation therapy (RT)[8]. The main principles in curative treatment of NSCLC are presented in **Figure 4**.

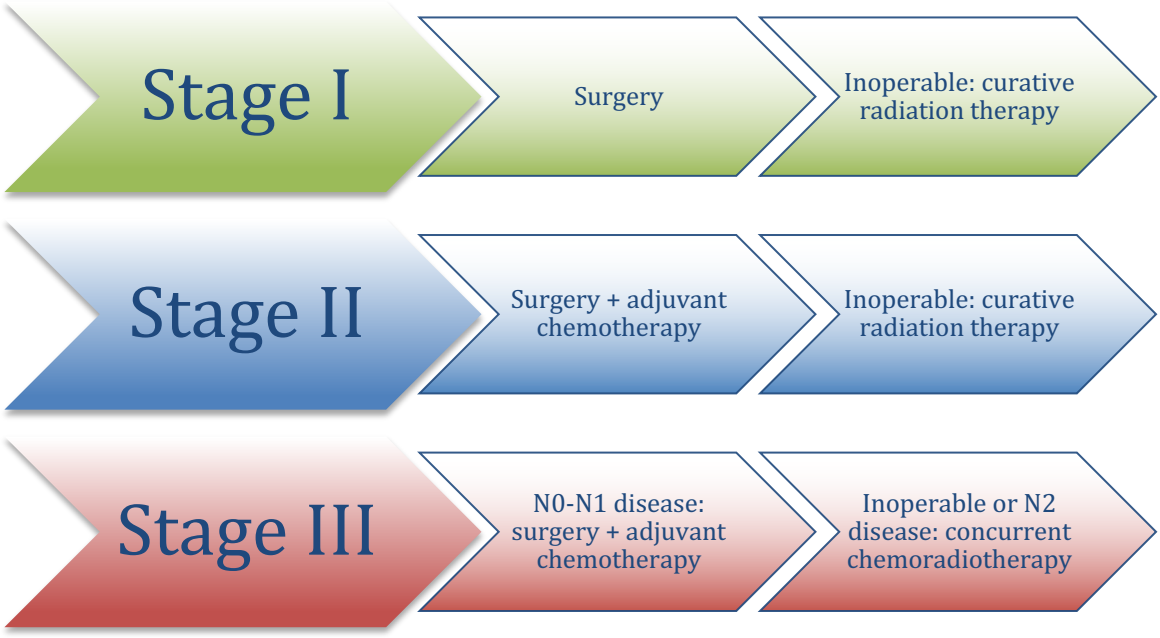

Figure 4 *Curative treatment strategy according to disease stage (Kaja Skjefstad)*

For stage I-II disease video-assisted thoracoscopic surgery (VATS) is the preferred surgical method due to fewer intra-operative complications and less post-operative pain[8]. Lobectomy is the most frequent surgical procedure performed in NSCLC resection and involves removing the tumor affected lobe. Other surgical options include sublobar resection, including segmentectomy and wedge resection, and pneumonectomy[50]. To ensure a thorough pTNM, essential for deciding adjuvant treatment, sampling or dissection of mediastinal lymph nodes is always performed[8]. If positive surgical margins are detected, re-resection or post-operative radiotherapy (PORT) is applied to obtain complete tumor remission. PORT is, however, only indicated after non-pneumonectomy procedures[8]. Stereotactic ablative radiation therapy (SABR) has emerged as a potential curative treatment alternative for early stage NSCLC patients. Studies have demonstrated significant local tumor control, and the treatment involves minimal side-effects[51,52]. Results from the Nordic SPACE (Stereotactic Precision and Conventional radiotherapy Evaluation) study, randomizing between SABR and conventional RT, indicated SABR as the primary treatment for inoperable stage I NSCLC[8,53].

Adjuvant therapy includes radiation and/or chemotherapy. PORT is administered when indicated (above section), and has in scheduled doses (< 54 gray, Gy) also shown a survival benefit in patients with N2 disease[54]. Adjuvant chemotherapy is recommended for surgically resected stage II and IIIA patients, but not for stage I disease or following curative radiotherapy. Surgery is an option for selected IIIA patients[39].

According to the Norwegian national guidelines, adjuvant chemotherapy as four cycles of cisplatin and vinorelbine is administered to patients < 70 years with ECOG 0-1, within eight weeks after curative surgery[8]. Patients > 70 years, but with a "biological age" < 70 years, may also benefit from chemotherapy[55]. Hence, they should always be considered for adjuvant chemotherapy if indicated. Neoadjuvant therapy is only indicated when the tumor is located to the sulcus superior (Pancoast-tumor)[8].

Stage III patients are a heterogeneous group. Thus, the treatment is tailored according to tumor characteristics including size (T-), the extent of nodular affection (N-), and other established prognostic factors, mentioned in the first section of this chapter. Concurrent chemoradiation is the best documented potentially curative treatment option for non-operable stage IIIA and IIIB patients. It is administered as 2 Gy x 30-33 with two platinum based chemotherapy cycles during radiation therapy[8].

Most patients, regardless of clinical NSCLC stage, relapse within 4 years after curative treatment[8]. The extent of pathologic lymph nodes is an important predictor for early disease recurrence[56].

1.1.6.2 Treatment of non-curable NSCLC

Stage III patients with concomitant negative prognosticators and stage IV patients, are offered palliative and life-prolonging treatment. This includes the majority (aprox. 70%) of patients diagnosed with, and treated for, NSCLC. Histological classification and subtyping of the NSCLC tumor is of outmost importance when deciding treatment strategy, and is performed

using small biopsy and cytology specimens as tumor resection is contraindicated in the palliative setting. It is pivotal to differentiate between adenocarcinoma and squamous cell carcinomas as all non-squamous cell carcinomas should be tested for EGFR mutations and ALK-translocations due to widely available targeted therapy[8].

Chemotherapy is one of the cornerstones in treating advanced NSCLC, and is the primary treatment in patients with advanced squamous cell carcinoma. In Norway, carboplatin/vinorelbine is the recommended platinum-doublet, due to less toxicity compared with other carboplatin-doublets (carboplatin/emcitabin, carboplatin/pemetrexed). It is administered for 3-4 courses in patients with ECOG status 0-2[8]. By disease progression after first line treatment with chemotherapy, second line chemotherapy treatment (Docetaxel) is recommended for patients in adequate performance status (ECOG 0-1). Further, conventional RT is an excellent symptom relieving treatment option for these patients. Thoracic radiation may significantly reduce tumor induced cough, chest pain, dyspnea and swallowing disorders, improving the patient's quality of life[57]. Palliative radiation is also used in the management of brain and bone metastases[8].

In Norway, the frequency of EGFR mutation positive patients is approximately 8 %, with a higher frequency in female patients[58,59]. The first line therapy for patients with activating EGFR-mutations is a tyrosine kinase inhibitor (TKI); erlotinib, gefitinib or afatinib. A TKI (crizitonib) is also first-line treatment in the 3-6% of non-squamous NSCLC patients holding ALK-mutations[60]. These TKIs have remarkable impact on clinical outcome[61-63]. Unfortunately, tumors will eventually develop resistance against the TKIs, thus most patients will relapse within 1 year[24]. Erlotinib is approved as second-line treatment in patients with disease recurrence, regardless of mutation status. Chemotherapy is also recommended as second-line treatment in patients with EGFR mutations and progressing on EGFR-TKI first-

line treatment. Re-introducing first-line treatment after relapse is also an option for patients with an initial good treatment response prior to disease recurrence[8].

Due to recent developments in immunotherapy, all NSCLC patients are tested for the expression of programmed cell death protein ligand 1 (PD-L1). Based on recent reports, monotherapy with programmed cell death protein 1 (PD-1) inhibitor pembrolizumab is recommended as first-line treatment in patients with PD-L1 expression >50%, without ALK- or EGFR mutations[8,64,65]. Further, the PD-1 inhibitor nivolumab and the PD-L1 inhibitor atezolizumab, have recently been recommended as second-line treatment in PD-L1 positive NSCLC patients with ECOG 0-1[8,60]. Patients relapsing after first-line immunotherapy may benefit from second-line platinum-based chemotherapy[60].

Patients treated for advanced NSCLC are closely monitored and controls are usually performed every 6th week, or more often if indicated.

1.2 Tumor biology

The Hallmarks of cancer, published by Hanahan and Weinberg in 2000 and 2011 (revised version), were suggested as common features characterizing the initiation and progression of tumorigenesis in malignant cells[66,67]. These steps include maintaining proliferative signals, evading growth inhibition, replicative immortality, resisting cell death, enabling invasion and metastasis, inducing angiogenesis, reprogramming cellular metabolism, tumor promoting inflammation and avoiding immune destruction (**Figure 5**)[66,67].

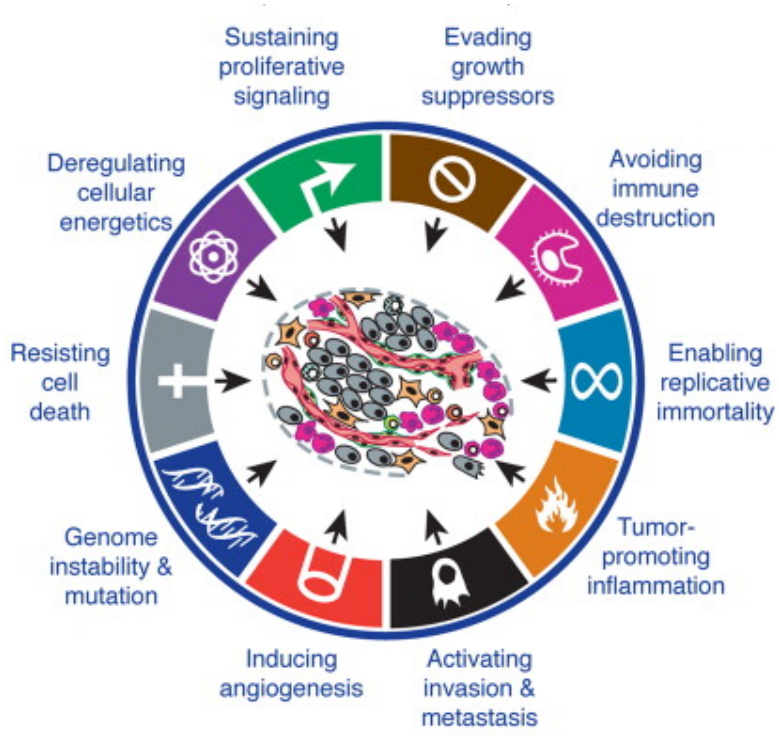

Figure 5 *An illustration encompassing the hallmarks and enabling characteristics of cancer. Reprinted from "Hallmarks of Cancer: The Next Generation"[66], modified by Kaja Skjefstad. Permission to reuse obtained from Elsevier.*

For these events to take place, genomic instability, often as a result of DNA damage, is necessary. The DNA strand may be assaulted by carcinogens (e.g. tobacco smoke), ionizing radiation, reactive oxygen species and viruses[68]. The DNA repair machinery usually prevents serious consequences of DNA lesions, e.g. interaction with replication and transcription, by cell-cycle arrest, regulation of DNA replication and repair of DNA damage[68]. Defects in the DNA repair system may cause genomic instability and subsequent somatic mutations followed by progressive morphological and molecular abnormalities and malignant transformation[28,68]. Driver mutations are essential in this process, affecting signaling pathways responsible for cell proliferation and survival. Further, it activates oncogenes and inactivates tumor-suppressor genes, facilitating tumor cell growth[69]. Additional factors are however necessary for a genetic assaulted cell to develop into a malignant cell[70]. Steroid hormones and their receptors represent important proliferation and growth promoting

signaling pathways that may be exploited to promote tumorigenesis and sustain proliferative signaling in an abnormal cell. Further, interactions between sex steroid hormones and the tumor surrounding stromal microenvironment may promote cancer, and also initiate the development of neoplastic growth[71].

1.2.1 Tumor microenvironment

The cancer cells are not solely responsible for the development and progression of neoplastic growth. The tumor microenvironment (TME) has emerged as an important contributor to the hallmarks of cancer (**Figure 5**), and hence as an accessory to disease progression. The TME consists of different cell types in extracellular matrix (ECM) including endothelial cells, fibroblasts, macrophages and immune cells[72]. These cells are often designated as tumor surrounding stromal cells.

The complex and not yet completely revealed multistep transformation of a "normal" stromal cell into a so-called tumor-associated stromal cell, also designated as a "corrupted" stromal cell, appears to be the consequence of interactions between malignant cells and surrounding stromal cells. This usually results in establishing supportive conditions for the malignant cells to thrive and eventually to spread[73]. Extracellular proteinases, including matrix metalloproteinases (MMP), are important drivers in this transition, promoting TME changes during tumorigenesis[74]. MMPs are produced by stromal cells infiltrating the tumor, and distinct stromal cells produce subsets of MMPs[74]. Cancer-associated fibroblasts (CAFs) are acknowledged as key promotors in tumor progression and metastasis. CAFs constitute a mixture of various fibroblasts with different origins. Upon activation, they express pro-invasive factors, including transforming growth factor-β (TGF-β), and mitogenic factors, including insulin-like growth factor-1 (IGF-1), to create a tumor promoting environment benefiting cancer cell growth and progression[73,75,76].

There is a continuous and bilateral cancer-stromal cross-talk conducted by signaling molecules, including microRNAs (miRNAs), facilitating the transformation from a healthy stromal environment to a TME[77]. Further, various miRNAs are suggested to be involved in tumor angiogenesis, one of the cancer hallmarks, ensuring cancer cell survival[77,78]. CAFs are also well-known contributors to the angiogenic process in tumorigenesis[79]. Taking into account the essential interaction between malignant cells and the surrounding stromal cells for tumor progression, stromal cells emerge as a potential therapeutic target. A series of clinical trials have been conducted using TGFβ-inhibitors, also in NSCLC[80]. Results indicate that TGFβ inhibition may promote disease control[81]. It is pivotal to detect reliable predictive biomarkers that may aid patient selection for this treatment.

When elucidating the potential for new treatment strategies in NSCLC, it is inevitable to include the TME and its potential. Interactions between NSCLC cells and tumor surrounding stroma has been suggested as a possible contributor to EGFR-TKI resistance, accentuating their significance in NSCLC treatment development on the long term[82]. In light of this, we have throughout this thesis included both tumor epithelial cells and tumor associated stromal cells in our NSCLC TMAs for investigating biomarker expression and their potential prognostic impact.

Epithelial to mesenchymal transition (EMT) is a differentiation process where an epithelial cell may partly or completely acquire mesenchymal characteristics. It represents an important step in tumor progression, tumor cell motility and metastasis[72]. EMT is induced through a complex signaling cascade within the tumor microenvironment, resulting in activation of transcription regulators including Snail, Slug, ZEB1 and ZEB2[83]. Recent reports have suggested steroid hormone receptors including ERα, ERβ and PR to affect this process by interacting with epithelial-related transcription factors including FOXA1 and midkine (MK)[83-85]. Further, induction of EMT by estrogen signaling was recently reported to be associated

with the development of therapeutic resistance towards immune-mediated cytotoxicity and chemotherapy[86]. miRNAs have also been suggested as important regulators of EMT in lung cancer pathogenesis with reports assigning miRNAs either anti-EMT or pro-EMT properties[87,88]. Inhibiting or reversing the EMT process is a sought after therapeutic modality in preventing tumor progression, but also the development of therapy resistance[86,89]. This further substantiate the importance of elucidating the TME and its components when investigating a biomarkers prognostic significance. However, due to the methods applied in this thesis, EMT was not the focus of this work.

A major part of the tumor environment is the immune system. It is acknowledged as a "double agent" in cancer progression, by both promoting and inhibiting cancer cell growth. Estrogens and their receptors are one of the factors regulation the immune response of the innate and the adaptive immune system, by enhancing or suppressing transcription of immune related genes[90]. Cancer associated chronic inflammation promotes tumor growth by innate immune cells releasing growth factors, angiogenic factors and survival factors to the microenvironment [66]. The immune system exerts tumor suppression in a multi-step process involving recognition and elimination of tumor cells by effector T cells. Initiation of this multi-step process relies on the activation of T-cells by stimulatory and co-stimulatory signals. Co-inhibitory signals, known as immune-checkpoints, are also important modulators of T-cell activation by suppressing T-cell reactivity (**Figure 6**)[91]. The tumors have developed several defense mechanisms avoiding immune destruction, as described by Hanahan and Weinberg in 2011 [66]. One crucial trait in immune resistance involves immune-checkpoints, that normally has an immune-inhibitory role in the self-tolerance of immune responses[92]. Cytotoxic-T-lymphocyte-associated antigen 4 (CTLA-4) and PD-1 are two inhibitory immune-checkpoint receptors (**Figure 6**) that have been meticulously investigated in regards to cancer immunotherapy[92].

This has resulted in the implementation of CTLA-4-inhibitors in cancer treatment[93]. More recently, the inhibition of PD-1 and its ligand PD-L1 has also been implemented in cancer treatment, including the treatment of advanced or metastatic NSCLC disease, as described in chapter 1.1.6.2[8,64].

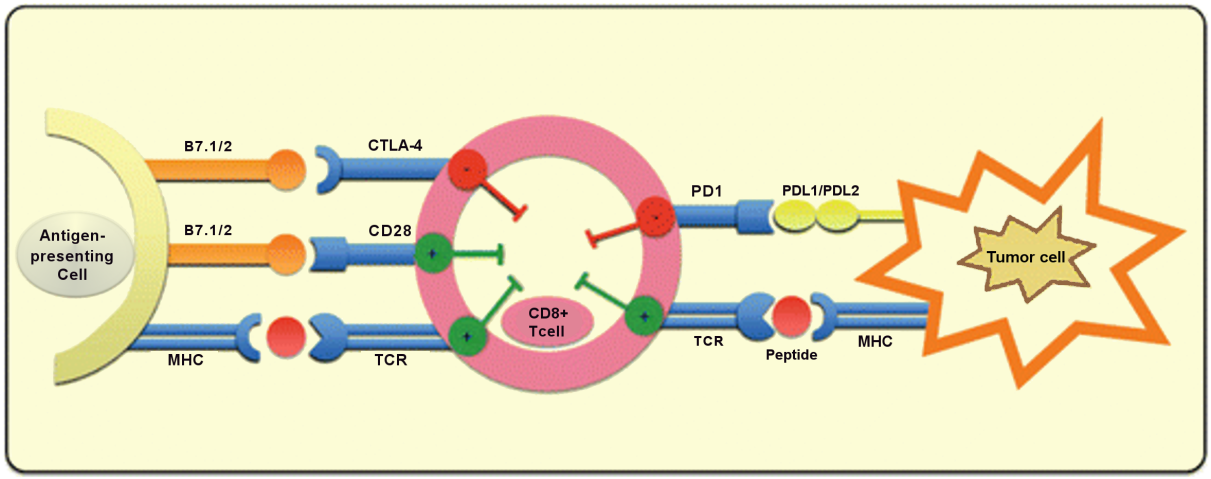

Figure 6 *Activation of CD8+ T-cell by co-stimulatory (green) and co-inhibitory (red) signaling molecules. Co-inhibitory signals, known as immune-checkpoints, are CTLA-4 and PD-1. Reprinted from "Immunotherapy in NSCLC: A promising and Revolutionary Weapon"[94]. Permission to reuse obtained from Springer.*

1.2.2 Sex steroid hormones in cancer

The female sex steroid hormones and their receptors involvement in carcinogenesis have been known for decades, and their contribution to the development of neoplasia was early proposed[95]. In hormone-dependent carcinogenesis, endogenic and exogenic sex steroid hormones promote proliferation and growth of tumor cells. Additionally, they contribute to important hallmarks including angiogenesis, apoptosis, tumor-promoting inflammation, migration and invasion[68]. Their tumor growth-promoting qualities is the target of current hormone therapy, by inhibiting their respective receptor. This is now an established therapeutic method in breast (estrogen receptor alfa, ERα), uterine (progesterone receptor, PR) and prostate cancer (androgen receptor). In non-reproductive tissues, sex steroid hormone signaling is involved in multiple mechanisms promoting tumor growth by affecting the tumor

microenvironment, the immune system and the tumor metabolism[71]. Co-regulators are essential in SHRs transcriptional activity, and the steroid receptor co-activators (SRC), are the most renowned. Abarrant SRC activation is believed to be an important contributor to hormonal-dependent carcinogenesis[96]. Additionally, steroid hormones have shown to induce double stranded DNA lesions, resulting in subsequent genomic aberrations[68]. High levels of estrogens are associated with an increased risk of developing breast cancer, and estrogen-induced DNA damage is proposed as one of the contributing factors[97]. Further, there are established evidence that ERs up-regulate genes involved in promoting metastatic disease including matrix metalloproteinases (MMPs) and metastasis-associated protein (MTA1)[98]. These findings demonstrate how sex steroid hormones and dysregulated activation of SHR may contribute to neoplastic development and growth through several mechanisms.

PR in endometrial cancer, and ER and PR in breast cancer, are established biomarkers and targets for hormone therapy[99,100]. Based on results from studies in Norwegian breast cancer patients, approximately 85% present with ER positive tumors, and 65% express PR[101]. Aromatase (AR), the rate-limiting enzyme in estrogen formation, is associated with breast cancer growth by producing large amounts of estrogen locally, increasing the estrogen-mediated cancer cell growth. This discovery led to the successful development of AR-inhibitors, the first-line hormonal therapy of SHR-expressing breast cancer patients today[102]. The vast majority of hormone receptor positive patients are offered adjuvant treatment with an AR-inhibitor (post-menopausal patients), or the selective estrogen receptor modulator tamoxifen (pre-menopausal patients)[103]. Adjuvant endocrine therapy has improved survival among breast cancer patients immensely, and hormone receptor-positive breast cancers are associated with a favorable prognosis compared to hormone-receptor, or triple negative tumors[103]. Regarding the treatment of high and moderate differentiated uterine tumors, high-dose progestin is used[104,105].

1.2.3 Sex steroid hormones and their receptors

Steroid hormones are the second largest group of hormones after peptide hormones. They arise from a common series of pathways with cholesterol as the joint precursor. Production and secretion are located to the adrenal cortex, ovaries and testicles, and regulated by the hypothalamic-pituitary axis. Additionally, circulating precursors are converted to biological active steroids by tissues including placenta, fat, brain, skin and liver[106]. Steroid hormones are dependent on transporting proteins, due to their hydrophobic nature. Sex-hormone binding globuline (SHGB) and albumin are examples of proteins binding to, and subsequently transporting, steroid hormones to their target destination[106].

According to their physical behavior, five groups of steroid hormones are generally recognized: glucocorticoids, regulating carbohydrate metabolism and manage stress; mineral corticoids, maintaining blood pressure by regulating the body's salt balance; vitamin D, essential in the calcium homeostasis; androgens, essential in fertility as well as the regulation of secondary sex characteristics in males and females; female sex steroids progesterone and estrogens, key roles in reproduction and the normal development of female genitalia and sexual characteristics [106]. Molecularly, estrogens regulate cell growth, differentiation and development. Hence, abnormal ER signaling is associated with various pathological conditions including obesity, cardiovascular disease, inflammation and even cancer[107]. Steroid hormones bind to their respective SHR, a member of the nuclear receptor family. Binding of ligand usually initiates a conformational change of its receptor, enabling binding to nuclear receptor responsive elements on the DNA strand. This will result in a transcriptional regulation. The transcriptional regulation is executed by recruited co-regulators and co-factors targeting steroid hormone-related genes. The genetic and cellular effects differ between cell- and tissue expression, depending on which co-regulators and co-factors are activated in the given receptor-expressing cell[108].

1.2.3.1 Estrogen

There are three estrogenic hormones: estron, estriol and 17β-estradiol (E2), the latter being the most potent. Estrogens are predominantly produced in ovaries of pre-menopausal women, while in men and post-menopausal women, it is synthesized from circulating precursors (androstenedione and testosterone) to E2 by the cytochrome P450 aromatase enzyme[109]. This local conversion takes place in adipose tissue, breast and brain. Estrogens exert their biological functions through binding to either of the two estrogen receptors, ERα or ERβ. The receptors are encoded by genes localized on different chromosomes, ERS1 and ERS2, and several mRNA splice-variants of each receptor have been described (**Figure 7**)[107].

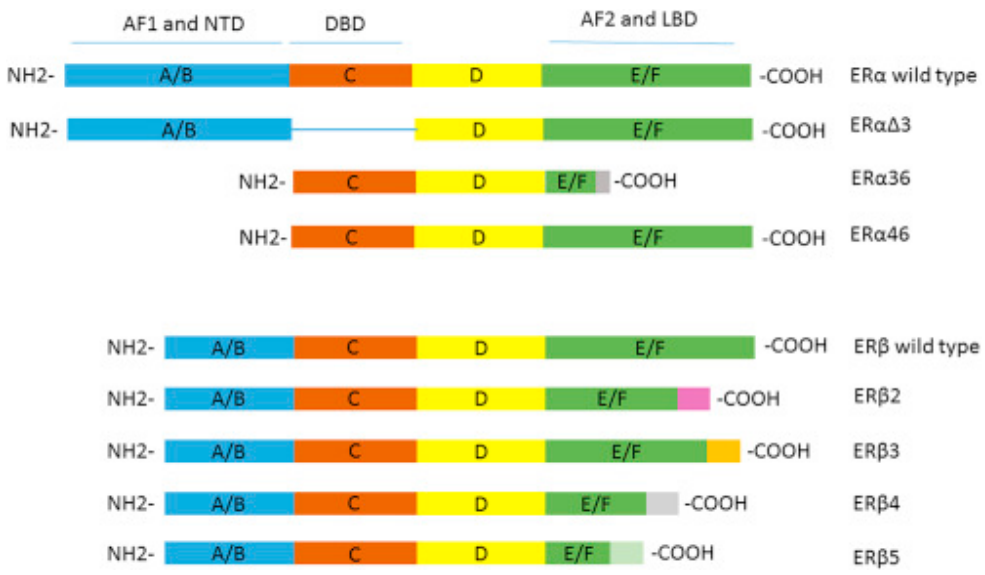

Figure 7 *ERα, ERβ and their respective isoforms. Functional domains include NH2-terminal domain (blue), DNA-binding domain (orange) and ligand-binding domain (green). Reprinted from "Estrogen receptor alpha and beta in health and disease" [109]. Permission to reuse obtained from Elsevier.*

The designations ERα wild type and ERβ wild type indicates the full-length receptors (**Figure 7**). Both receptors consist of three functional domains serving different roles in receptor signaling. The DNA binding domain (DBD) is responsible for recognizing, and binding to,

the estrogen-responsive elements (ERE) on the DNA strand upon receptor activation. The COOH-terminal ligand-binding domain (LBD) is responsible for binding ligand and the NH2-terminal domain (NTD) is involved in regulating target genes by its ligand-independent activation function, AF1[109]. The DBD is the most similar domain between ERα and ERβ, while both LBD and NTD differ in their structure. This provides receptors with distinct roles in transcriptional activity and has led to the development of subtype-specific ligands[109]. Upon ligand-binding, the receptor initiates genomic estrogen effects, by binding directly to ERE on the DNA strand, inhibiting or promoting mRNA translation. ERs may also regulate transcription ligand-independently, through interaction with other DNA-bound transcription factors, or through receptor activation by extracellular signals including epidermal growth factor (EGF) and IGF-1[107,110]. ERs are also localized outside of the cell nuclei, in the cytosol or the plasma membrane[111]. Here, they interact with receptors including EGFR, HER-2, IGFR-1 and several signaling molecules including members of the ras/MAPK cascade[111]. The receptors differ in tissue distributions. ERα is mainly expressed in reproductive tissue, including ovary and uterus, breast, kidney, bone, adipose tissue and liver. ERβ is expressed in the ovary, central nervous system, cardiovascular system, lung, male reproductive organs, prostate, and the immune system[109].

1.2.3.2 Progesterone receptor

The progesterone receptors (PR) belong to the ligand-activated nuclear transcription factors, and contain the same functional domains as ERs (**Figure 7**). PRs are expressed as two isoforms, PR-A and PR-B. They are translated from the same gene, by alternate use of two promotors[112]. One-hundred-and-sixty-four amino acids located in the N-terminal region of PR-B, separates the two isoforms from identical structures[113]. This small inequality, however, renders a distinctively different gene regulation between the receptors[114]. Ligand binding of progesterone (P4) or progestin, a synthetic compound mimicking the activity of P4, activates

the receptor[115]. Through associating with a wide range of coregulatory molecules they bind to progesterone responsive elements (PRE) on the DNA strand, influencing progesterone-specific gene promotors[112,113]. PR-A is a transcriptional regulator of steroid hormone expression, repressing PR-B and ER expression. PR-B is predominantly a transcriptional activator, and is responsible for the proliferative progesterone-mediated effects observed in mammary glands[112].

PR-A is primarily localized in the cell nucleus. PR-B shuttles between the cell cytoplasm and nuclei, mediating transcriptional activity, but also non-nuclear signaling through modulating cell signaling pathways including Src/Ras/MAPK and PI3 kinase/Akt[112]. Ligands may also bind to membrane progesterone receptors (mPRs) and mediate rapid, cell-surface signaling[116]. PR-A and PR-B are co-expressed in equivalent levels in PR expressing cells. Malignant tissue shows changes in isoform expression, with a predominant expression of either PR-A or PR-B, depending on malignancy[113].

1.2.3.3 Steroid hormone-associated miRNAs

miRNAs are a class of endogenous, small (19-25 nucleotides), noncoding RNA gene products that regulate gene expression by targeting mRNAs for degradation or translational repression[117]. miRNAs are expressed as precursor RNAs which are subsequently processed in the nucleus and cytoplasm by cellular enzymes like Drosha and Dicer to yield mature miRNAs[118]. Research has revealed abnormal miRNA expression in several types of cancer, suggesting that these small noncoding RNA genes play a crucial role in cancer pathogenesis in the human body[119]. Several X chromosome-linked miRNAs contribute to a sex-specific regulation of immune-related genes, and are thought involved in an enhanced immune surveillance against cancer[71]. miRNAs are involved in the complex gene network controlled by estrogens and several studies have indicated that miRNAs are controlled by estrogens in hormone-related cancer cells including breast and uterine cancer[107].

1.3 Endocrinology related biomarkers in NSCLC explored in this thesis

Reports have indicated a possible role of steroid hormone receptors in the development of NSCLC, and suggested them as potential prognostic biomarkers[120-124]. Selected biomarkers incorporated in this thesis are presented in the following three paragraphs, with emphasis on their potential role in NSCLC.

1.3.1 Paper I: Progesterone receptor

Progesterone plays a crucial role in fetal lung development by increasing VEGF expression and surfactant proteins[125]. After birth, progesterone receptors are present in lung tissue. They also play a role in tissue changes by inflammatory diseases as well as in lung tumorigenesis[126]. However, there have been conflicting reports regarding expression levels and their possible role in lung malignancies[120]. Little is known about PRs molecular function in NSCLC, but studies have reported the presence of progesterone synthesizing enzymes in NSCLC tumors, indicating a local production of progesterone[126]. PR has been reported as a positive prognosticator in NSCLC, and progesterone treatment of tumor xenografts have demonstrated growth inhibition and induction of apoptosis[120,122].

1.3.2 Paper II: Estrogen receptor α, estrogen receptor β and aromatase

There are mounting evidence that estrogen can up-regulate genes involved in cell proliferation in NSCLC (e.g. cyclin-D1 and c-myc) and other important estrogen-responsive genes, including PR[127].

Both ERα and ERβ are expressed in normal lung tissue as well as NSCLC[128]. ERβ is widely distributed throughout the body, including the lungs, and has been detected both in a nuclear and cytoplasmic location in NSCLC cells. Wild-type ERβ is suggested as the primary ER expressed in lung tumors, mediating ER-related biological effects[129]. A recent report by Pelekanou et al.[130], however, reports ERα as the predominant ER expressed in NSCLC. Both

genomic, and non-genomic ER signaling is reported in NSCLC cells[131,132]. ERβ expression is associated with increased mitogenic qualities in NSCLC cells, by enhancing transcription of growth promoting genes, both *in vitro* and *in vivo*[123,131,133]. Further, ERβ is involved in extracellular matrix organization and stromal-epithelial cross-talk, and is associated with an increased expression of the metastasis–associated matrix metalloproteinases (MMPs)[134]. ERβ has been proposed as an unfavorable disease prognosticator in NSCLC[120]. Many efforts to explore the prognostic significance and the potential therapeutic effects of ERs in NSCLC have been attempted, important milestones are presented in **Table 2**.

Reports have indicated that the majority of intra-tumor E2 in lung cancer, as in breast cancer, is produced locally, i.e. by malignant cells, due to increased levels of AR[135,136]. Biologically active AR has been found in lung tumor tissue resected from both female and male patients, and correlates with high concentrations of intra-tumoral estrogen[127,135].

Table 2 *Presenting important advancements in the potential of hormone-targeted treatment in NSCLC. Adapted from "Targeting the estrogen pathway for the treatment and prevention of lung cancer"[127], modified by Kaja Skjefstad. Permission to reuse from Lung Cancer Management as agreed by Future Medicine Ltd.*

Milestones in research on ERs prognostic relevance in NSCLC	
Year	Event
2002	ERβ is found to be highly expressed in the majority of NSCLC cases[123]
2005	First report of non-genomic ER signaling and connections between the ER and EGF receptor pathways are reported[133]
2009	Hormone replacement therapy is found to be associated with increased lung cancer mortality[22] The first pilot study of antiestrogen therapy in combination with gefitinib was completed and demonstrated safety and potential efficacy[137]
2011	Breast cancer patients receiving adjuvant antiestrogen therapy have reduced lung cancer mortality risk[138] ERβ is reported as a prognostic marker for poor prognosis in early stage NSCLC[136]
2012	First demonstration that antiestrogens and aromatase inhibitors can prevent lung tumor formation in vivo[132]
2013	The first Phase I trial of antiestrogen therapy with erlotinib in advanced NSCLC is completed with promising results[139]

1.3.3 Paper III: miR-143 and miR-145

Since their discovery in 1993, over 2500 human miRNAs have been recorded in miRBase, a searchable database for published miRNAs and their target predictions[140]. These small non-coding RNAs regulate gene expression post-transcriptionally. Furthermore, miRNA dysregulation plays an important role in cancer development[141]. miRNAs are involved in important steps of tumorigenesis by regulating cell cycle progression, apoptosis and

autophagy, and tumor cell motility, invasion and metastasis[142]. Their interactions with the TME promotes additional hallmarks of cancer (chapter 1.2.1).

Mounting evidence appoints miRNAs as important prognosticators in NSCLC, and due to their precise gene regulation, they are considered potential therapeutic targets. The development of miRNA-based therapeutics represents a novel strategy in the battle against cancer[143]. A phase 1 clinical trial of miRNA replacement therapy in thoracic cancer patients was recently completed, with promising results[144].

The miRNA cluster 143/145 consists of two miRNAs, miR-143 and miR-145, both regarded as tumor suppressors which target a number of genes involved in tumorigenesis[145]. They are often reported as down-regulated in several types of cancers including head and neck, breast and lung[145,146]. Sex steroid hormones and their receptors have been proved to regulate the expression of miRNA cluster 143/145 in various malignancies, including breast and ovarian cancers[145,147,148]. Interestingly, miRNAs have emerged as important components in estrogen-induced genetic alterations in various malignancies[107].

1.4 Predictive vs prognostic biomarkers

A biological marker is defined as an objective, quantifiable characteristic of a biological process that can be measured accurately and reproducibly[149]. In cancer research, it is essential to separate between predictive and prognostic biomarkers. A prognostic marker is a biomarker statistically associated with a patient's outcome. A predictive marker, on the other hand, is statistically associated with a certain treatment response, thus it may be used for selecting treatment for a patient. In NSCLC, EGFR, ALK and PD-L1, as described in chapter 1.1.3.2, 1.1.6.2 and 1.2.1, are characterized as predictive markers by predicting chemotherapy or targeted treatment response[150]. More than 100 prognostic biomarkers for lung cancer have been published, yet none have hitherto been widely implemented in the clinical treatment

setting[151]. NSCLC therapy including chemotherapy, RT and targeted therapy, shows different treatment outcome in females vs males, and several studies have proposed gender as a predictive biomarker[5]. Results are, however, conflicting, thus gender is so far not acknowledged for guiding therapeutic choices[5].

2 AIMS OF THIS THESIS

This thesis aimed to elucidate the potential prognostic role of endocrinology-related biomarkers in NSCLC and to determine their anticipated gender-related impact. Since anti-hormonal drugs are widely available, and steroid hormones have been shown to contribute in lung cancer development, we wanted to investigate steroid hormones and associated miRNAs as possible prognostic markers in NSCLC. By assessing marker expression in tumor cells, tumor associated stromal cells and metastatic lymph nodes (**paper III**), we wanted to provide a better understanding of tumor biology, growth and the cell-type specific biomarker expression.

Specific aims:

1. To elucidate the prognostic impact of PR in tumor epithelial cells and tumor associated stromal cells (**paper I**)
2. To examine the prevalence and the prognostic role of ERα, ERβ and AR in NSCLC (**paper II**)
3. To explore the prognostic significance of the steroid hormone related miRNA cluster miR-143/miR-145 in primary tumors and metastatic lymph nodes (**paper III**)

3 MATERIALS AND METHODS

3.1 NSCLC tissue samples

3.1.1 Patient cohort

In **paper I** and **paper II**, our original cohort was used. As described in the respective papers, it consisted of 335 NSCLC patients who underwent surgical resection for clinical stage I-IIIA disease at the Nordland Central Hospital (NS), Bodø and the University Hospital of North Norway (UNN), Tromsø from 1990 through 2004[152,153]. In 2013, we expanded our cohort with additional 218 patients after including resected patients from 2005 through 2011. Thus, **paper III** included analysis of survival data from the third and most recent update. Consequently, 553 resected patients with stage I-IIIB NSCLC, adequate tissue samples and complete medical records were included (follow-up data as of October 1st 2013), as described in **paper III**. We retrospectively collected clinical, demographic and histopathological data, this is presented in **Table 3** (details for our original cohort of 335 patients are presented in **paper I** and **paper II**). In addition, tissue specimens from 143 of 172 patients with metastatic lymph nodes (N+) were eligible for inclusion in the database, as their specimens were available and adequately paraffin-embedded.

For **paper I** and **paper II**, pathological staging was done according to the 7th edition of the UICC TNM classification. The tumor specimens resected prior to 2010, was originally staged according to the 6th edition of the UICC TNM classification. These were restaged and reviewed according to the 7th edition upon its implementation in 2010. Histological classification and subtyping was done on the basis of WHO guidelines of 2004. For **paper III**, pathological staging and histological classification and subtyping were updated to conform the recent 8th UICC TNM classification and the 2015 WHO guidelines, respectively[31,40].

During the time span 1990-2011, 633 NSCLC patients with stage I-IIIB disease, were surgically resected at NS and UNN. Exclusion criteria and number of patients excluded are presented in **Figure 8**.

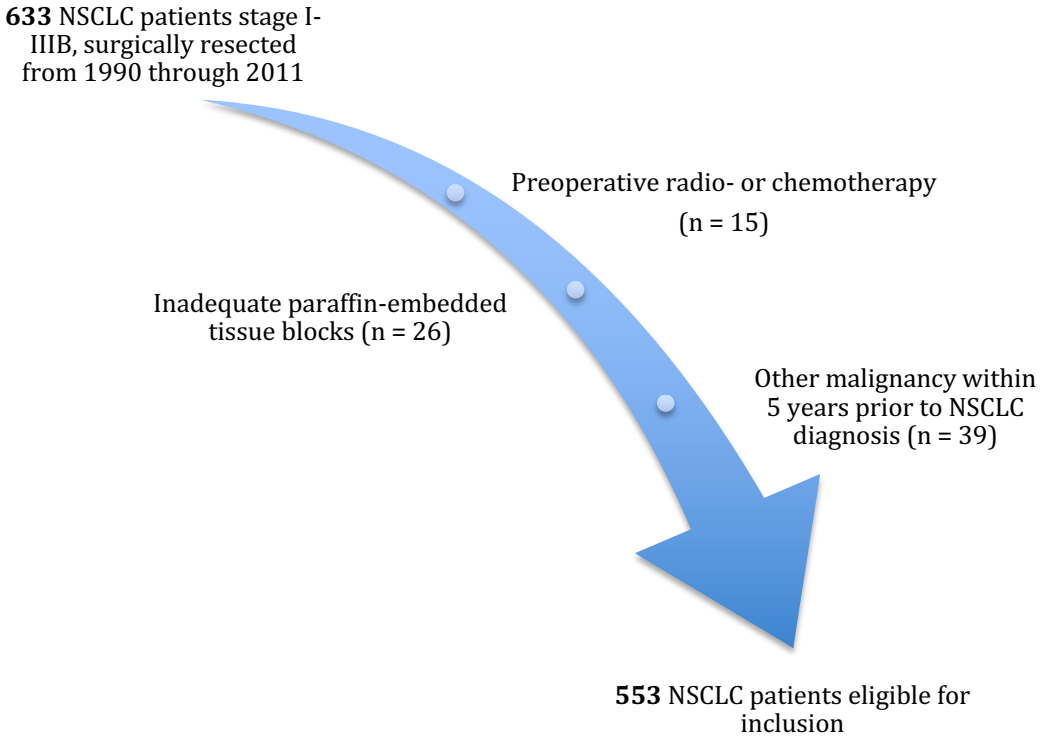

Figure 8 *Exclusion criteria applied in our study*

Table 3 *Clinical and pathological variables as predictors of disease-specific survival (DSS) in NSCLC patients (univariate analyses; log-rank test; N=553, 180 female and 373 male patients, respectively)*

All patients					**Female patients**				**Male patients**			
	N(%)	**5 year DSS (%)**	**Meadian DSS (mo)**	**p**	**N(%)**	**5 year DSS (%)**	**Median DSS (mo)**	**p**	**N(%)**	**5 year DSS (%)**	**Median DSS(mo)**	**p**
Age				0.656				0.637				0.827
≤65	234 (42)	58	127		77 (43)	62	190		157 (42)	56	98	
>65	319 (58)	58	NR		103 (57)	65	NR		216 (58)	44	88	
Sex				**0.025**								
Female	180 (33)	64	190									
Male	373 (67)	55	91									
ECOG perf. status				**0.009**				0.400				**0.020**
0	324 (59)	63	235		112 (62)	67	NR		212 (57)	60	235	
1	191 (34)	52	71		56 (31)	60	127		135 (36)	48	51	
2	38 (7)	52	NR		12 (7)	55	NR		26 (7)	50	NR	
Smoking				0.069				0.732				0.060
Never	21 (4)	50	21		11 (6)	64	189		10 (3)	33	18	
Present	350 (63)	62	235		115 (64)	67	NR		235 (63)	59	235	
Previous	182 (33)	52	84		54 (30)	58	NR		128 (34)	49	57	
Weightloss				0.971				0.603				0.637
<10%	498 (90)	58	190		163 (91)	63	190		335 (90)	56	91	
≥10%	55 (10)	59	NR		17 (9)	68	NR		38 (10)	54	98	
Surgical procedure				**< 0.001**				**0.024**				**< 0.001**
Wedge/Lobectomy	411 (74)	64	235		148 (82)	68	190		263 (70)	61	235	
Pulmonectomy	142 (26)	42	30		32 (18)	42	37		110 (30)	42	29	
Margins				0.105				0.088				0.431
Free	506 (91)	59	190		166 (92)	65	190		340 (91)	56	98	
Not free	47 (9)	47	57		14 (8)	51	64		33 (9)	45	47	
Tstage				**< 0.001**				**0.009**				**<0.001**
1a	14 (3)	93	NR		5 (3)	100	NR		9 (2)	89	NR	
1b	71 (13)	79	NR		30 (17)	82	NR		41 (11)	77	NR	
1c	95 (17)	64	190		33 (18)	66	NR		62 (17)	63	235	
2a	135 (24)	57	88		35 (19)	65	NR		100 (27)	54	83	
2b	73 (13)	48	47		28 (16)	60	NR		45 (12)	40	40	
3	104 (19)	56	NR		36 (20)	60	NR		68 (18)	54	98	
4	61 (11)	31	21		13 (7)	23	NR		48 (13)	36	19	
Nstage				**< 0.001**				**<0.001**				**<0.001**
0	379 (69)	70	235		132 (73)	74	NR		247 (66)	67	235	
1	118 (21)	36	35		23 (13)	42	47		95 (26)	35	27	
2	56 (10)	23	21		25 (14)	30	35		31 (8)	16	15	
Pathological stage				**< 0.001**				**<0.001**				**<0.001**
I	232 (42)	74	235		78 (43)	81	NR		154 (41)	70	235	
II	185 (33)	59	114		61 (34)	66	NR		124 (33)	56	91	
IIIA+B	136 (25)	28	21		41(23)	29	36		95 (26)	27	17	
Histology				0.241				0.431				0.125
SCC	307 (56)	64	235		77 (43)	71	NR		230 (62)	61	235	
ADC	239 (43)	52	73		100 (56)	59	190		139 (37)	46	57	
Other[a]	7 (1)	67	NR		3 (1)	50	11		4 (1)	75	NR	
Vascular infiltration				**< 0.001**				**0.040**				**<0.001**
No	453 (82)	62	235		136 (76)	68	190		317 (85)	60	235	
Yes	97 (17)	38	35		42 (23)	49	47		55 (14)	25	22	
Missing	3 (1)				2 (1)				1 (1)			

Note: Bold numbers indicate P<0.05. Abbreviations: ADC, adenocarcinoma. ECOG perf.status, Eastern Cooperative Oncology Group performance status. N, number. NR, not reached. Nstage, Nodal stage. Mo, months, SCC, squamous cell carcinoma. Tstage, Tumor stage. [a]Includes adenosquamous carcinoma, large cell carcinoma, and carcinoma not otherwise specified.

3.1.2 Tissue microarray

In the aftermath of Kononen et al.[154] started producing quality tissue microarrays (TMAs) in 1998, the technology has obtained an important role in laboratory diagnostics and is now considered essential in translational research. TMA facilitates a high-throughput molecular profiling of tumor tissue, providing information from up to 1000 specimens in a single TMA series staining[155]. TMA construction typically involves extracting representative tissue cylinders from paraffin-embedded or formalin fixed tumor material, and transferring them to a recipient block. They are meticulously transferred in a specific checkerboard pattern so that each core may be easily identified and matched with clinical data from the respective patient[154]. Depending on study aim, methods such as *in situ* hybridization (ISH) or IHC may be applied on the TMAs to analyze expression patterns or cellular distribution of specific biomarkers. Prognostic TMAs are the most common used TMAs, and allows us to research the association between biomarker expression and prognosis[156]. The representativity of TMAs compared with whole tissue specimens is satisfactory[157].

3.1.2.1 Construction of TMA

TMA construction performed in our group has previously been described in detail[158]. The construction methodology is presented in **Figure 9**. Prior to TMA construction, tissue preparations are made: 1) formalin fixation, 2) dehydration and 3) embedding in paraffin wax[159]. Formalin fixation causes the formation of inter and intra-molecular cross-links between amino acids, which in turn prevents degradation of the tissue and tissue components[160]. Paraffin-embedded whole tissue specimens from all of the lung cancer patients were investigated thoroughly by an experienced pathologist.

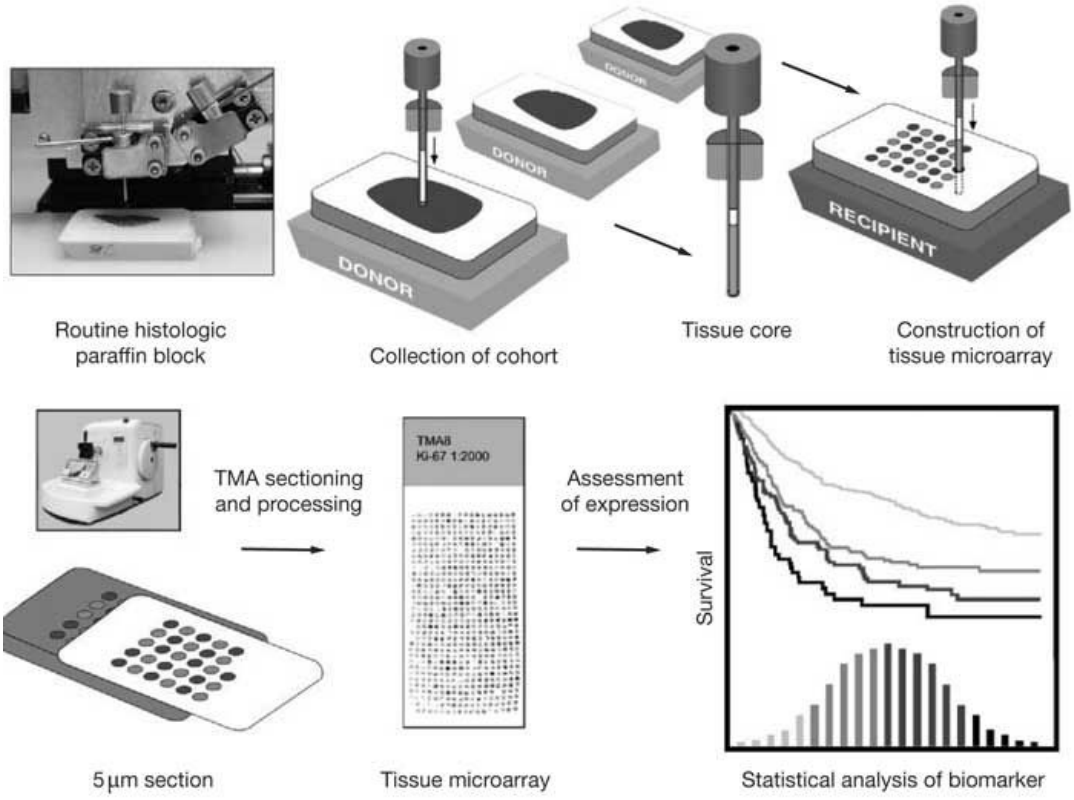

Figure 9 *TMA construction. As previously described, two cores from tumor epithelial cells and two cores from tumor surrounding stroma were marked in each cellular compartment in order to guide sampling for the recipient microarray blocks [152]. Using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD, USA) with a thin-walled biopsy needle and stylet, cylindrical cores (diameter 0.6 mm) were extruded from the representative areas and transferred to a predrilled, recipient block [152]. For ISH and IHC analyzes, the blocks were cut into 4-µm thin sections using a Micron microtome (HM355S) and stained with probes or specific antibodies for biomarker expression profiling [152,153]. Figure adapted from "Technology Insight: Identification of biomarkers with tissue microarray technology" [161]. Permission to reuse obtained from Nature Publishing Group©.*

The sample quality, cellular content, and histological subtype were carefully assessed, and the most representative areas of tumor epithelial cells and tumor surrounding stroma were identified[152,153]. For patients with N+ disease, two cores were extracted from the most eligible metastatic lymph node and included in the recipient block. Further, lung tissue far from the site of the tumor was collected and used as tissue staining controls. For **paper I** and **paper II**, eight blocks were constructed in order to include all of the collected cores from the tissue

samples. For **paper III**, twelve blocks of primary tumor tissue and three blocks of metastatic lymph node tissue were constructed.

3.1.3 Immunohistochemistry

Immunohistochemistry (IHC) is a laboratory technique used to detect cell or tissue antigen by means of specific antibodies binding to the antigens. Subsequently, the antigen-anibody complex is visualized by staining. This allows us to investigate the expression of several antigens, including proteins, amino acids and infectious agents. It is also an important tool in elucidating differential diagnosis, and is widely used in basal research[162]. Indirect IHC is the most common method and was used in this thesis. Staining procedures can be performed automated or manually. Both techniques were applied herein.

3.1.3.1 Antibodies

Antibodies are immunoglobulins constituting the principal effectors of our adaptive immune system. They are also extensively used for therapeutic, diagnostic and research purposes [163]. Antibodies can be subdivided into monoclonal antibodies (MAbs) and polyclonal antibodies (PAbs). MAbs are produced by one single B-cell clone, providing a homogeneity superior of PAbs. PAbs are produced from a span of different B-cell clones, causing a variation in antigen specificity. The homogeneity of MAbs ensure a highly specific binding to its corresponding epitope[163]. Antibodies used in this thesis are presented in **Table 4**. For supplementary antibody details, see "Experimental" **paper I** and **paper II**[152,153].

Table 4 *Overview of antibodies utilized in this thesis*

Antibody	Clone number	Host species and clonality	Producer	Concentration/dilution	Catalogue number
PR	1E2	Rabbit monoclonal	Ventana	Prediluted	790-4296
ERα		Rabbit polyclonal	Santa Cruz	1:100	SC-543
ERβ	PPG5/10	Mouse monoclonal	AbD Serotec	1:10	MCA1974s
AR		Goat polyclonal	Santa Cruz	1:100	SC-14245

3.1.3.2 IHC procedure

For manual staining (ERα, ERβ, AR), all sections were deparaffinized with xylene, and rehydrated with ethanol[153]. As described in **paper II**, antigen retrieval was performed by 1) placing the specimens in 0.01 M citrate buffer with pH 6.0 and 2) repeated microwave heating at 450 W for 10 minutes. To avoid non-specific staining, the tissue sections were incubated with 5% normal serum ABC kit (Vector Laboratories) and blocking solution to quench endogenous peroxidase activity[153]. For **paper I**, blocking of endogenous peroxidase was performed by the DAKO EnVision + System-HRP (DAB) kit (DAKO, Glostrup, Denmark) [152]. For **paper II**, incubation with a solution of 0.5% hydrogen peroxide for 10 minutes quenched the endogenous peroxidase activity[153]. After blocking, tissue samples and corresponding primary antibodies were incubated overnight. Subsequently all sections were incubated with a detection reagent consisting of a secondary antibody conjugated with an avidin/biotin complex[152,153]. According to the manufacturer's instructions, the Vectastatin ABC kit (Vector Laboratories) was used in this process. The primary-secondary antibody complex was detected in its cellular localization by adding a chromogen (here: DAB), causing

a brown staining (**Figure 10**). Finally, counterstaining with hematoxyline was performed to visualize cell nuclei[153].

For automated staining (PR), the Ventana Benchmark XT automated slide stainer (Ventana Medical Systems, Tucson, AZ, USA) was used[152]. The principles for automated staining requires the same multistep procedure as for manual staining, elucidated in the section above.

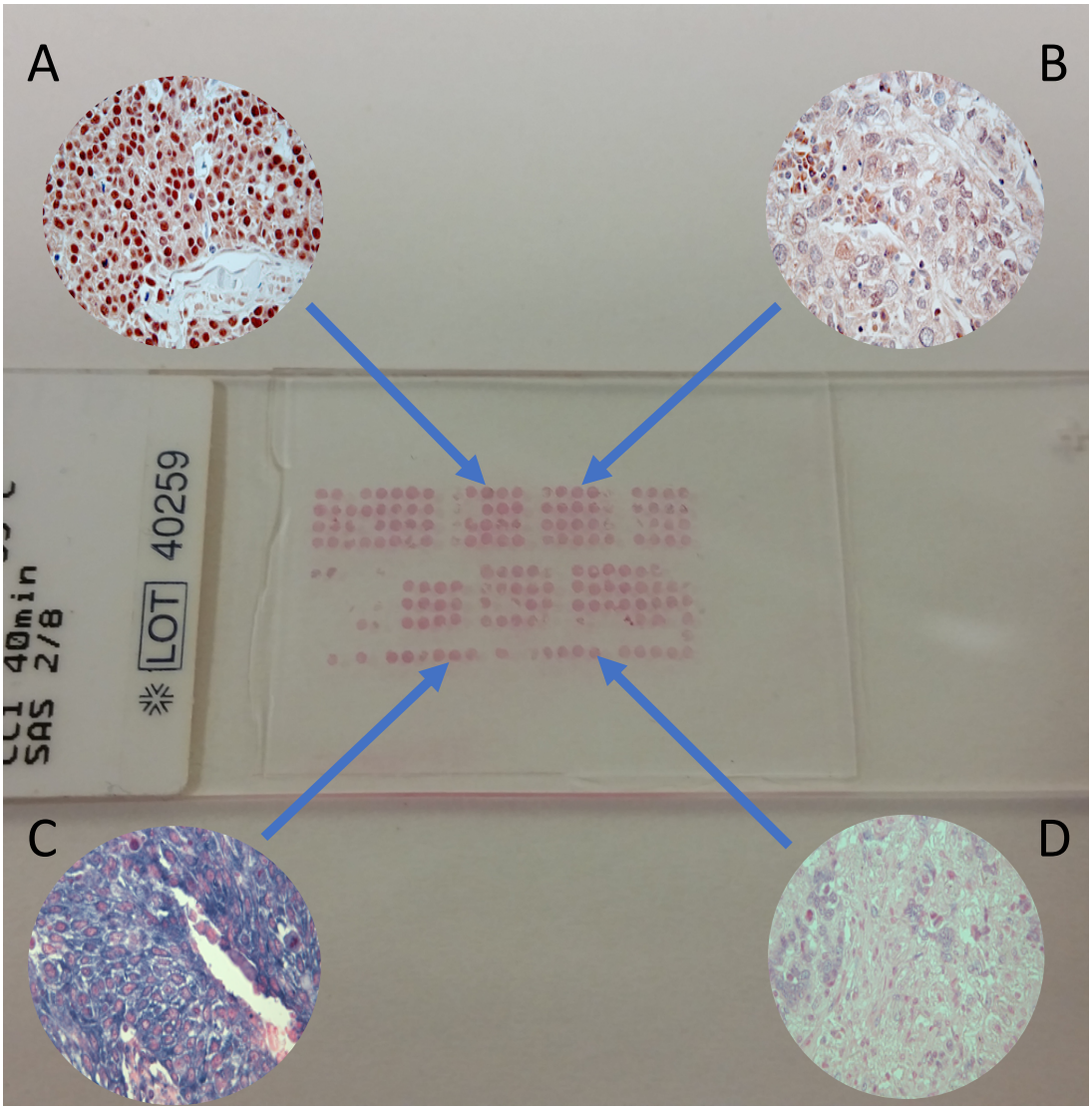

Figure 10 *Tissue microarray slide showing immunohistochemical staining of A) high ERβ expression, 400x magnification, B) low ERβ expression, 400x magnification, and staining visualized by in situ hybridization of C) high miR-143 expression, 400x magnification, D) low miR-143 expression, 400x magnification. (Kaja Skjefstad)*

3.1.4 *In situ* hybridization

ISH is a technique developed in the 60´s, with the aim of detecting specific nucleotide sequences such as DNA or RNA in tissue sections or individual cells. The technique involves binding of a labelled nucleotide probe to a specific DNA or RNA sequence of interest. Secondly, the hybridized probe and sequence may be visualized microscopically. Visualization method depends on probe labelling; antigen, fluorescent or other labels [164]. Chromogenic ISH (CISH) is based on the same chromogenic signal transduction as IHC, and allows visualization in an ordinary light microscopy. This is in contrast to fluorescence ISH (FISH), which is dependent on a fluorescence microscope[165]. CISH was the chosen method in this thesis. ISH allows detection of a biomarkers cellular origin[166]. This was of outmost importance as we aimed to elucidate the miRNAs roles in both tumor associated stroma, metastatic lymph nodes and primary tumor tissue.

3.1.4.1 ISH procedure

In **paper III**, miR-143 and miR-145 expression in NSCLC tissue was analyzed by ISH, applying TMA slides. ISH was performed using the Ventana Discovery Ultra Instrument (Ventana Medical Inc, Arizona, USA). For details regarding detection probes, reagents and buffer coats, see "Materials and methods", **paper III**. To ensure a high sensitivity level of the ISH method, protocol optimization was performed. This included 1) minimizing the risk of RNA degradation by using RNAse-free water in buffers and during sectioning, and applying protective equipment whenever handling slides and reagents; 2) testing of probe concentrations and demasking pretreatments in NSCLC tissue and 3) testing of hybridization temperatures for each probe (miR-143 and miR-145). Additionally, staining sensitivity was ensured by the use of positive (U6 snRNA control probe) and negative (scramble miR probe) controls. For further details on procedure and staining, see "Materials and methods", **paper**

III. Biomarkers visualized by ISH is presented in **Figure 10**, table C and D. miR probes used for detection of miR-143 and miR-145 is presented in **Table 5**.

Table 5 *miR probes used in this thesis*

miR	Probe	Catalogue	Producer	Concentration
miR-143	miR-143, -3p target probe	88068-15	Exiqon	10 nM
miR-145	miR-145, -5p target probe	38515-15	Exiqon	2.5 nM

3.1.5 Evaluation of staining

For PR, ERβ, AR, mir-143 and miR-145, the degree of biomarker staining was assessed by light microscopy. For ERα, the ARIOL imaging system (Applied Imaging Corp., San Jose, CA, USA) was used to manually score the IHC stained TMA slides on computer screen. Detailed method on loading and scanning of the slides is presented in **paper II** (Experimental; IHC scoring). For all biomarkers, both cytoplasmic and nuclear staining in tumor epithelial and tumor associated stromal cells was considered. All samples were independently scored by two pathologists (PR, by E.R. and S.A.S.) or one pathologist and a medical student, now medical doctor, trained by a pathologist (ERα, ERβ, AR, miR-143, miR-145, by S.A.S. and K.S.). The scoring of biomarker expression was performed semiquantitatively. Intensity of biomarker staining in neoplastic epithelial cells and tumor associated stromal cells was scored as: 0 = negative, 1 = weak, 2 = intermediate, 3 = strong. Tumor epithelial PR staining was heterogeneous, thus density in tumor epithelial cells was scored. Staining in neoplastic epithelial cells for ERα, ERβ, AR, miR-143 and miR-145 was homogenous, subsequently biomarker density was not scored. Due to staining heterogeneity in stromal cells, density was scored for ERβ, AR, PR, miR-143 and miR-145. This was performed in the following manner: 0 = no cells stained positive, 1 = 1-5% cells showed positive staining, 2 = 6-50% cells were positive, 3 = > 50% positive cells. ERα staining was

not observed in tumor associated stromal cells. In **paper III**, locoregional metastatic lymph nodes were stained and scored under the same condition as tumor and stromal tissue. Due to excessive stained lymphocytes, however, the stromal compartment of lymph nodes was not scored. For each patient, the mean score of duplicate cores was calculated. When both intensity and density was scored, the scoring values were combined and the mean score was calculated. Before evaluation of each biomarker, measures of quality controls were made between the scorers. This included 1) for ERα, ERβ, AR, miR-143 and miR-145; a thorough training of the medical student, now medical doctor (K.S.), by an experienced pathologist (S.A.S.), 2) a mutual agreement regarding the semi-quantitative scale was made, 3) clarification on which compartment to score for each biomarker and 4) several illustrative examples of each score was reviewed and compared with control tissue. After scoring, an inter-observer reliability test was performed to further ensure the quality of staining interpretation. The test results were found highly satisfactory for each biomarker, intra-class correlation coefficients (ICC) are presented for each biomarker in the respective papers.

3.1.6 Determination of cutoff values

For statistical analyzes on biomarker expression and its association with prognosis and survival, one needs to categorize the biomarker expression. Using a continuous scale provides more information and renders a more accurate expression profile. To translate this continuous variable into a clinical determination, it is however necessary to determine a cutoff point, thus stratifying patients into two separate groups[167]. Categorizing can be done by: 1) calculating the mean/median value and dichotomizing the patients by high/low expression, 2) biological determination or 3) the minimum p-value approach[168]. The cutoff was set at mean value for PR, ERα, ERβ, AR and miR-143. For miR-145, zero was the cutpoint that best differentiated between patient groups, subsequently this was the chosen cutoff. A complete list of cutoff values is presented in **Table 6**. By using the mean value, one ensures reproducible results and

minimize the risk of type I errors (false positive expression). The mean value, however, may not represent the biological relevant cutoff, thus increasing the chance of type II errors (false negative). For miR-145, based on the minimum p-value approach, zero was the chosen cutoff. As our study is hypothesis generating, choosing biomarker cutoffs based on a minimal p-value approach is appropriate.

Table 6 *Cutoff for each biomarker according to cellular compartment. NS, not scored.*

Paper	Biomarker	Cell compartment	Cutoff
Paper I	PR	Neoplastic epithelium	1
		Stroma	0.5
Paper II	AR	Neoplastic epithelium	1.69
		Stroma	1.52
	ERα	Neoplastic epithelium	1.80
		Stroma	NS
	ERβ	Neoplastic epithelium	2.3
		Stroma	1.67
Paper III	miR-143	Neoplastic epithelium	1.98
		Stroma	1.87
	miR-145	Neoplastic epithelium	0
		Stroma	0

3.2 NSCLC cell lines

For **paper III**, we performed a series of *in vitro* experiments to investigate the potential function of miR-143 and miR-145 in NSCLC tumorigenesis. We used five commercial cell lines, all purchased from American Type Culture Collection (ATCC). The cell lines and relevant data is presented in **Table 7**.

Table 7 *Lung cancer cell lines used in this thesis*

Name	Cell type	Organism	ATCC®
A549	Adenocarcinoma	Human	CCL-185™
H520	Squamous cell carcinoma	Human	HTB-182™
H460	Large cell carcinoma	Human	HTB-177™
H661	Large cell carcinoma	Human	HTB-183™
NL20	Non-tumorigenic bronchial epithelium	Human	CRL-2503™

3.2.1 *In vitro* experiments

Details regarding the experimental procedures for cell culture conditions, cell transfection and *in vitro* experiments, are presented in **paper III**. The basic principles for cell cultures include: 1) culturing of all cell lines in RPMI-1640 media with 10% fetal bovine serum and 1 x penicillin-streptomycin antibiotic mixture, 2) incubation at 37° C in humidified atmosphere with 5% CO_2 and 3) transfection of cell lines with miR-143 mimic and/or miR-145 mimic in combination with miR negative control. For transfection, the transfection reagent Lipofectamin® 2000 (Life Technologies, Waltham, USA) was used.

3.2.1.1 Proliferation assay procedure

Proliferation ability of transfected cells was evaluated by the real-time cell analyzer xCELLigence RTCA DP (ACEA Biosciences, San Diego, USA). Cells were seeded as quadruplicates into an E-plate, 5000 cells per well, and mounted in the RTCA DP instrument. Cell index, a unit reflecting cell growth, was denoted by the instrument and measured every 15 minutes the first 24 h. Subsequent measurements were made every 30 minutes for the remaining assay duration. Proliferation curves were calculated using RTCA software, version 1.2.1.

3.2.1.2 Migration assay procedure

For cell lines A549 and H661, migration abilities were assessed using ibidi^{TM} culture inserts (ibidi GmbH, Planegg, Germany). Transfected cell-suspension was added to inserts positioned into a 12-well tissue culture dish, one insert per well. The inserts consist of two 0.22 cm^2 silicone chambers with a 0.5 mm divider. Cells were left to adhere for 24 hours before inserts were removed. Cell migration into the 0.5 mm gap was calculated using TScratch, version 1.0 (CSElab, Computational Science and Engineering Laboratory, Switzerland) and imaging was performed at 0 and 20 hours.

3.2.2 Reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) is the gold standard in terms of nucleic acid quantification and allows amplification and quantification of both precursor miRNA and mature miRNA [169]. In 2011, Busk et al. developed a PCR method using DNA primers, yielding a more sensitive and specific quantification of miRNAs. In **paper III**, endogenous levels of miR-143 and miR-145 were quantified by RT-PCR according to Busk et. Al's principles [170]. Expression levels were quantified relative to the non-cancerous cell line NL20.

3.3 Statistical analyzes

Statistical analyzes performed in this thesis was solely executed with the statistical package IBM SPSS, versions 21-24 (IBM Corp., Armonk, NY, USA). The primary end-point was disease-specific survival (DSS), defined as time from surgery to lung cancer death. The last update for DSS was in October 2013. The Kaplan-Meier method was used when examining associations between biomarker expression and DSS in univariate survival analysis. Statistical significance between survival curves was analyzed by the log-rank test. All variables, both clinicopathological and biomarkers, with significant p-values in univariate analyzes were included in multivariate analyzes, for which the Cox Proportional Hazard model was used. Data was run in a backward conditional method with probability for stepwise entry and removal at 0.05 and 0.10. P-values < 0.05 were considered statistically significant, representing the probability of observing our results when the null hypothesis is correct. When running multivariate analyzes on female and male patients separate, the subgroups were firstly run in univariate analyzes to confirm significance. This was performed in **paper I-III**. Associations between marker expression and clinicopathologic variables were investigated by χ^2 test or Fisher's exact test. Spearman´s rank correlation coefficient was calculated for the correlation between biomarkers. Inter-observer reliability tests were

performed for each biomarker to ensure consistent interpretation of staining results. The intra-class correlation coefficient was good or excellent (range: 0.75-0.97).

The power, expressed as 1 – β, represents the probability of a study discovering a true association. Herein, β represents the type II error rate and depends on sample size as well as the size of effect the investigator is interested in[171]. One may calculate the sample size necessary to obtain a high probability of finding a true association, hence a small β[172]. For this study, original sample size calculations indicated that 300 patients had to be included in the study to achieve a power at 80% and an α-value at 5%. α represents the probability of type I error, and is often determined in advance of an analysis, standard value is 5%[172]. 5-year DSS for resected NSCLC patients is 60 %, and survival was the primary endpoint when calculating sample size. Biomarker expression that caused a 50% increase in hazard ratio was considered clinically significant, and the frequency of a given biomarker is typically around 35%. Calculations was done using PASS 2002, Number Cruncher Statistical Systems, Kaysville, Utah, USA.

3.4 Ethics

The herein presented study was approved by the Regional Committee for Medical and Health Research Ethics (REK) (protocol ID: 2011/2503) and The Norwegian Data Protection Authority. The majority of patients were diseased at the study initiation and the tissue specimens were more than 10 years old, subsequently REK deemed patient consent was not a prerequisite. Biomarker expression levels and patient details, including clinical and pathological data, was reported according to the REMARK guidelines[173].

4 MAIN RESULTS

4.1 Patient characteristics

Clinicopathological and demographic data for all 553 patients are presented in **Table 3**, chapter three. Of these were 295 patients diagnosed and treated at the University Hospital of North Norway (UNN), the remaining 258 were treated at Nordland Central Hospital (NS). Of the 553 patients, 407 died during the follow-up time, 223 died of lung cancer or causes related to lung cancer (e.g. pulmonary embolism). Recurrence rate was 47%, comprising 262 patients. 5-year survival for the end-points overall survival (OS), disease-free survival (DFS) and disease-specific survival were 46%, 71% and 58%, respectively. Corresponding median survival was 47, 115 and 190 months, respectively. No significant discrepancies between survival (DSS, OS) were noted between the treating hospitals. Median age at diagnosis was 67 years (range 28-85). The majority (67,5%) of patients were male, 32,5% were female. In the original cohort (335 patients), the largest histologic subgroup was SCC with 60% of cases, vs ADCs (39%). However, this difference was rather balanced in the updated material where SCCs comprised 48%, while ADCs accounted for 50% of all cases. For all 553 patients (original and updated cohort), histological subgroups were distributed as follows: 307 SCCs (56%), 239 ADCs (43%) and 7 others (1%). ADCs was the most common histological subtype in female patients (56%), while SCCs was the most frequent in males (62%). Only 4% of the patients were never-smokers, whereas 96% were present or previous smokers. The main surgical method was lobectomy (70%), and pulmonectomy as the second most frequent technique (26%). Only 4% underwent wedge resection. The majority of wedge resections were performed at UNN (82%). Despite a slight difference in frequencies of surgical methods and histological subtype, the two cohorts from Tromsø and Bodø were similar with regards to age, gender, WHO performance status (ECOG), smoking and adjuvant radio or chemotherapy

(**Table 8**). Adjuvant chemotherapy had not been introduced as a standard therapeutic option in Norway during the time span 1990-2004, thus the majority of patients receiving chemotherapy were treated in the period between 2005 to 2010.

Table 8 *Clinicopathological and demographic data from patients surgically resected at the University Hospital of North Norway (UNN) vs Nordland Central Hospital (NS)*

Variable	UNN	NS
Gender		
Female	98 (33%)	82 (32%)
Male	197 (67%)	176 (68%)
Age		
Under 65	127 (43%)	107 (42%)
Over 65	168 (57%)	151 (59%)
Histology		
SCC	146 (50%)	161 (62,5%)
ADC	143 (48%)	96 (37%)
Other*	6 (2%)	1 (0,5%)
Smoking		
Never	13 (4%)	8 (3%)
Present	182 (62%)	168 (65%)
Previous	100 (34%)	82 (32%)
Adjuvant radiotherapy		
No	244 (82,7%)	232 (90%)
Yes	50 (17%)	26 (10%)
Missing	1 (0,3%)	
Palliative radiotherapy		
No	228 (77%)	210 (81%)
Yes	67 (23%)	48 (19%)
Chemotherapy		
No	237 (80%)	213 (83%)
Yes	58 (20%)	45 (17%)
ECOG perf.status		
Normal	161 (55%)	163 (63%)
Slightly reduced	107 (36%)	84 (33%)
In bed < 50%	27 (9%)	11 (4%)
Survival		
Median DSS (months)	235	190
Median OS (months)	54	43
5-year DSS (%)	59	57
5-year OS (%)	48	43

Abbreviations: ADC, adenocarcinoma. ECOG perf. status, Eastern Cooperative Oncology Group performance status. SCC, squamous cell carcinoma
*includes adenosquamous carcinoma, large cell carcinoma and carcinoma not otherwise specified (NOS)

4.2 Paper I

Sex steroid hormone receptors (SHRs) and their signaling targets are essential in the development of normal lung parenchyma. Furthermore, their importance in lung cancer pathogenesis has been declared in several reports (chapter 1.3.1. and 1.3.2.). In this study, we investigated the prevalence and the prognostic role of PR in neoplastic epithelial cells and tumor surrounding stromal cells in resected tumors from 335 NSCLC patients[152]. Demographic, histopathological and clinical variables for all 335 patients are presented in **paper I** and **paper II**.

4.2.1 Biomarker expression

Applying IHC technology and light microscopy, the majority of PR expression was observed in the cell nucleus. PR expression above mean value, which was the chosen cutoff value, was observed in 34.3% and 32.2% in the tumor and stromal compartment, respectively.

4.2.2 Survival analyzes

In univariate analysis, we revealed that positive PR expression was associated with a favorable prognosis for all patients (P=0.005). Interestingly, we found that female patients with PR positive tumor cells had a worse outcome compared to male patients with PR positive tumor cells (P=0.003). This finding revealed a gender specific role of PR in NSCLC. A combination of high PR expression in tumor cells and low expression in stromal cells yielded an even worse prognosis in the female patient group (P<0.001), than either of the variables independently. In the multivariate analysis, PR expression in stromal cells emerged as an independent positive prognosticator in all patients (HR 1.74; P=0.007). For female patients, epithelial tumor cell PR expression was a negative prognosticator in the multivariate analysis (HR 3.46; P=0.001)[152].

4.3 Paper II*

To further elucidate the significance of SHRs in NSCLC, we aimed to investigate the expression profile and prognostic relevance of ERα and ERβ, and the rate-limiting enzyme in estradiol synthesis: the aromatase enzyme (AR), in our NSCLC cohort.

4.3.1 Expression and correlations

IHC staining of ERα was predominantly nuclear, wild-type ERβ (ERβ1) was observed in both nucleus and cytoplasm and AR staining was primarily cytoplasmic in epithelial cells in the primary tumor (PT) and stromal cells. Epithelial and stromal biomarker expression was homogenously distributed. ERα was not observed in stromal cells[153]. ERα expression was significantly increased in PT, compared to non-neoplastic tissue (cytoplasmic, P=0.001: nuclear, P=0.010). This was not observed for ERβ and AR. Biomarker expression was significantly correlated in PT and stromal cells for AR (r=0.46, P<0.001) and ERβ (r=0.21, P<0.001). We found weak, but highly significant, correlations between AR expression in PT and biomarkers related to angiogenesis, previously investigated by our research group (VEGFR; r=0.277, P<0.001 and PDGFA; r=0.239, P<0.001). This was also found for ERβ expression in PT (VEGFR; r=0.238, P<0.001 and PDGFD; r=0.280, P<0.001).

4.3.2 Survival analyzes

In survival analyzes, ERα had no impact on 5-year survival. High ERβ expression emerged as a negative prognosticator for female patients in univariate analysis (P=0.010). Further, AR was associated with an unfavorable prognosis in all patients (P=0.017). High expression of ERβ in female patients and AR in all patients were both independent negative prognosticators for DSS (HR: 3.03, P=0.005; HR: 1.55, P=0.017) in multivariate analysis[153].

* Due to the first author´s inattentiveness, **Figure 3** in this paper is unfortunately not the correct figure that was intended for publication. A corrigendum with the appropriate figure is attached, after **paper II**.

4.4 Paper III

Based on studies reporting an interesting cross-talk between a subset of miRNAs and the estrogen-signaling pathway (chapter 1.2.3.3.), we wanted to investigate the prognostic relevance of the tumor-suppressor miRNA cluster miR-143/145 in our NSCLC cohort, and to see if we could correlate these biomarkers with our previously investigated SHRs.
This study was performed on our updated patient cohort consisting of 553 NSCLC patients, surgically resected at The Nordland Central Hospital in Bodø and the University Hospital of North Norway, Tromsø. Clinicopathological and demographic characteristics are herein listed in **Table 3**, and in **paper III**. We explored biomarker expression in tumor cells, tumor associated stroma and locoregional lymph nodes by *in situ* hybridization in our TMA samples. Further, we performed a series of *in vitro* experiments to evaluate the functional role of miR-143 and miR-145 in NSCLC tumorigenesis.

4.4.1 Expression and correlations

Microscopic evaluation of ISH staining revealed that miR-143 expression in tumor and stromal cells was primarily cytoplasmic, while miR-145 was mainly observed in the cell nucleus. Our results regarding biomarker expression evaluated by ISH and RT-PCR were conflicting. Scoring of ISH staining showed a significantly upregulated biomarker expression level compared to non-malignant tissue as opposed to RT-PCR where miR-143 and miR-145 were found to be downregulated in four lung cancer cell lines, relative to a non-cancerous cell line. Correlation analyzes revealed several interesting links between the miRNA cluster and

previously investigated SHRs including PR, AR and ERβ. miR-143 expression in lymph nodes was positively correlated with stromal AR expression (r=0.494: P<0.001), and inversely correlated with PR expression (-r=0.453: P<0.001). Further correlations included: miR-143 in primary tumor (PT) with cytoplasmic ERβ in PT (r=0.215: P<0.001) and miR-145 in PT with nuclear ERβ expression in PT (r=0.212: P<0.001)

4.4.2 Functional cell line studies

Four independent lung cancer cell lines (A549, H520, H460, H661) were used to evaluate the cancer cells ability to proliferate and migrate upon transfection with miR-143, miR-145 or both. We showed that miR-143 and miR-145 inhibit migration in lung cancer cell lines (A549, H661). The major trend in transfecting cell lines with miR-143 and/or miR-145, was a decrease in proliferative capacity. An increase was, however, seen in the H520 cell line after transfection with miR-143.

4.4.3 Survival analyzes

We report stromal miR-143 expression as an independent prognosticator of favorable outcome in female patients (univariate analysis P=0.011; multivariate analysis HR: 0.53, P=0.019). miR-145 expression in stromal cells was associated with an improved DSS for male patients in univariate analysis (P=0.013) and multivariate analysis (HR:0.58, P=0.021).

5 DISCUSSION

5.1 Materials and method

5.1.1 Study designs

This thesis includes different study designs and is based on renowned research methods (TMA, IHC, ISH, western blot, cell cultures and RT-PCR) in order to thoroughly investigate our aims and purposes. Study designs include observational cohort study (**paper I, II and III**) and experimental study (**paper III**). Throughout this study, the importance of transparency in regards to methods and result interpretation has been pivotal. This, to ensure reproducible and reliable results accessible for comparison and further evaluation by research colleagues nationally and internationally.

5.1.1.1 Observational cohort study

Observational studies include cohort, cross-sectional and case-control studies, and the common denominator is the lack of intervention from the investigator performing the study. Cohort studies are most commonly associated with determining incidence and prognosis of a certain condition. It may be conducted prospectively, by following a group of people over a certain time, or retrospectively, by analyzing already existing data for a certain patient group[174]. Since the data has already been acquired, a retrospective study is considerably economical to perform compared to a prospective study. A huge disadvantage is the data quality. Demographic and clinical data is collected from hospital journals, and there are considerable variations in the amount and quality of information provided for each patient. Smoking history, in particular, represents a challenge. It seldom exists exact tobacco data making calculation of pack years difficult.

5.1.1.2 Cell line studies

Human cancer cell lines are widely used as experimental models to investigate the biology of human cancers. Advantages by using cell lines includes the possibility for the investigator to perform interventions and observe effects. In addition, cell lines have the ability of limitless replication providing unlimited material access[175]. Further, they possess the hallmarks of cancer cells (chapter 1.2), enabling the investigation of tumor growth, invasion and metastasis[176]. Cancer cell lines constitute a pure tumor cell population, in the absence of tumor associated stromal- and inflammatory cells, and vascular compartments. This represents both an advantage, as it provides a thorough tumor cell profiling, and a disadvantage, as stromal and inflammatory cells are a huge part of the tumor microenvironment involved in tumorigenesis (chapter 1.2.1)[177]. This is a challenge when comparing results from arrays based on tumor tissue collected from patients, with arrays based on cancer cell lines.

5.1.2 Patient cohort

We have a large patient cohort of NSCLC patients (original cohort: 335, updated cohort 553), surgically resected at UNN and NS. Our exclusion criteria, presented in chapter three, were applied to minimize the risk of bias. Since another malignancy within 5 years of a lung cancer diagnosis may affect the patient response to cancer treatment, patients with such events were excluded from our cohort. Similarly, may neoadjuvant treatment (radio or chemotherapy prior to surgery) affect the tumor microenvironment by activating immune related cells and molecules, causing a host response. Consequently, patients with neoadjuvant treatment were also excluded. Our updated cohort consists of 32.5% female patients. During the last decade there have been observed an equalization of lung cancer incidence between male and females, and the sex ratio today is close to 1:1, male:female[7]. However, geographically conditioned variations between lung cancer incidence by sex, exists[6]. Recent years have also revealed a

shift in the most frequent histological subtype from SCCs to ADCs. Our database has a majority of SCCc, due to a higher frequency of SCCc in our early material (1990-2004). These factors may contribute to a lack of representability in our results, and validation in an external cohort with recently collected tissue samples and clinicopathological variables is necessary. The collection of data and inclusion of patients for this study was done during a time span over 20 years. Several changes and improvements in lung cancer treatment were made during these years, with the implementation of adjuvant chemotherapy in 2005 as the most important. This represents a critical confounder in any retrospective study collecting data over time, including ours. Diagnostic criteria and tumor classification have also evolved during the follow-up period. All our tumor specimens have been thoroughly examined by an experienced pathologist, both prior to TMA inclusion and in conjunction with reclassification, whenever new classification criteria have been implemented. This ensures continuity and minimizes the risk of inter-observer variations in tumor classification.

Even though lung cancer is highly associated with smoking, an increasing group of patients diagnosed with lung cancer are never-smokers, especially in the Asian countries[178]. It is estimated that 15% of lung cancer cases arise in never-smokers making this one of the leading causes of cancer-related mortality worldwide[15]. Very few of our patients were classified as never-smokers (4%), this is, however, representative in the western part of the world.

5.1.3 TMA

The most prominent advantage of TMA technology is the saving of cost, time and tissue. Staining and analyzing of whole-tissue sections (WTS) is a time-consuming process and involves cutting and preparing histologic sections from hundreds of patients. Immunostaining would be performed in batches in order to handle the enormous amount of tissue samples, reducing staining sensitivity and specificity caused by batch-to-batch variability[179]. Subsequently, a pathologist would examine all stained slides and evaluate staining level and

identify the respective tumor cell compartments[179]. This is a time-consuming process making standardization of staining evaluation difficult. TMA allows a high-throughput analysis and visualization of molecular markers in hundred to thousand tissue samples simultaneously[154]. Only one slide from each recipient TMA block is needed per investigated biomarker, saving reagents and tumor tissue. All representative tumor cores have been carefully selected by a pathologist, prior to TMA construction. This selection acts as a quality control, making it possible for non-pathologists to reliably evaluate biomarker staining in the tissue samples, as performed for **paper II** and **paper III**[155,179]. Because one single TMA slide may contain up to 800 specimens, the tissue magnitude per biomarker staining is limited. This allows for staining of all tissue samples simultaneously under the exact same conditions, accounting for the standardization characterizing TMA technology[155].

Preservation of tissue after removal from the human body is challenging. Proteins, DNA and RNA will immediately start degrading, problematizing sectioning and secondly microscopic observation. Fixation of tissue samples prevents degradation and autolysis, hence minimizing the time from tumor resection to fixation is pivotal[160]. Several studies have shown that the assessments of important biomarkers are confounded by delayed fixation, with dramatic consequences for patient care[180,181]. The quality of fixation also depends on the tissue size: when removing a complete organ, a lung for instance, it takes time for the formalin to penetrate the lung tissue. This may cause cells in the middle of the organ to degrade pending formalin penetration. Subsequently, tissue architecture and antigen epitopes alter, causing variations in biomarker staining and staining interpretation. This kind of sampling variability in protein expression was demonstrated by Permuth-Wey et al.[182] in ovarian cancer. Based on their findings they suggest sampling for TMA construction should be performed in the periphery of tumor blocks, due to its optimal fixation location. All our tumor specimens were thoroughly examined by an experienced pathologist prior to TMA construction, ensuring the

inclusion of well-preserved and well-fixated primary tumor and tumor surrounding stromal tissue.

A much-debated challenge in TMA technology is whether the 0.6 mm tissue cores are representative for the heterogeneous tumor architecture. However, studies performed early in the TMA era proved a concordance of 90-95% when comparing biomarker expression in TMAs with WTS[183,184]. A more recent study by Pøhl et al.[185] elucidated the reliability of IHC-determined biomarkers in NSCLC using TMA technology. They concluded that biomarkers can be adequately assessed using 1 or 2 biopsy samples (1mm diameter). However, the optimal number for biomarkers with functional properties was between 1 and >4 cores. A common presumption in these kind of comparative studies is that WTS represent the "gold standard" in regards to tumor heterogeneity and biomarker expression. This assumption may not be correct as one single WTS only contain minor parts of the tumor volume, and therefore only represent a very small part of the tumor[185]. Biomarker heterogeneity may vary immensely between different biomarkers. If the biomarker is measured with error due to heterogeneity, this may affect the power of the study and it is known that increased heterogeneity diminishes the ability to detect a specific effect size[186]. To counteract this and increase the power of the study one can increase the number of cores per tumor specimen[186]. We used cores of 0.6 mm, and aimed to obtain at least four cores per patient. At the same time, a large number of patients were included in our cohort to further ensure reliable results. Additionally, the majority of biomarkers investigated in this thesis showed homogenous expression, thus TMA was considered a reliable and well-fit method.

5.1.4 IHC

Methodological challenges with IHC may be categorized in two: 1) challenges occurring prior to staining (pre-analytic), 2) challenges related to staining (analytic). The pre-analytic challenges are related to tissue processing. This includes tissue degradation, delayed tissue

fixation, under-fixation, over-fixation and inconsistent fixation[159]. Delayed fixation and under-fixation has been discussed in chapter 5.1.3. Over-fixating the tissue will complicate the demasking process and subsequent antigen retrieval in IHC, increasing the chance of false negative staining. IHC is a multi-step process and several aspects including demasking, antigen retrieval method and blocking of unspecific binding and endogen peroxidase activity may affect the consecutive staining evaluation. Means to ensure high quality results have been discussed in detail throughout chapter 3.1.3.

5.1.4.1 Antibodies

Choosing an appropriate antibody is pivotal in order to create reliable IHC analyzes. Only antibodies that were approved for IHC analyzes on paraffin-embedded tissue by the manufacturer, were used in this thesis. We used both monoclonal and polyclonal antibodies, as described in chapter 3.1.3.1. The antibodies where selected based on extensive literature reviews, performed by experienced laboratory technicians. Advantages and disadvantages of mono- vs polyclonal antibodies are presented in **Figure 11**.

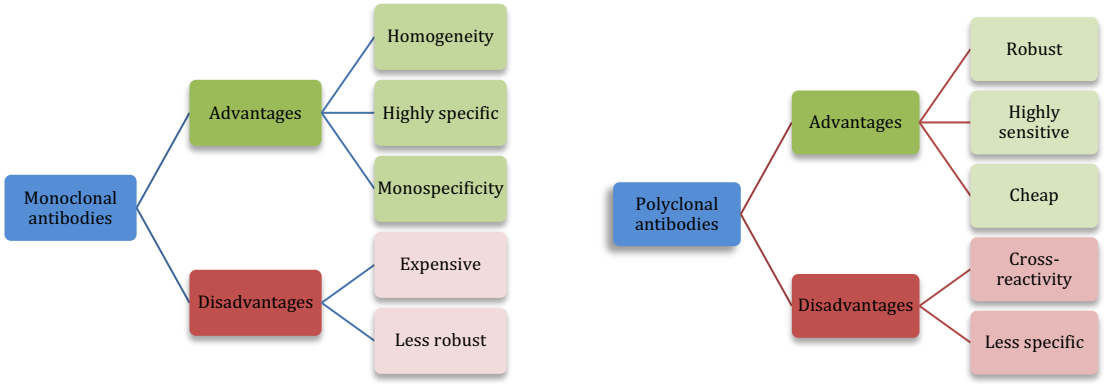

Figure 11 Advantages and disadvantages using monoclonal vs polyclonal antibodies *(Kaja Skjefstad)*

5.1.4.2 IHC controls

Validation of reagents and quality control of protocols and staining, play a crucial role in IHC. This is to secure the validity and reproducibility of the results[159]. Antibody specificity is usually ensured by western blot. For **paper I** (PR), western blot validation was performed by the manufacturer (Ventana Medical Systems). For **paper II** (ERα, ERβ, AR), we performed western blot analysis in order to validate the specificity of the primary antibodies. Positive and negative controls were used to further secure antibody specificity and a proper staining technique. Ovary and placenta were used as positive controls for ERα, ERβ and AR; pancreas and liver constituted negative controls. Negative controls were stained in absence of the primary antibody. This, to assess the presence of non-specific staining. To ensure specificity, it was important to perform the control staining simultaneously with the test tissue staining. PR staining was performed using the Ventana Benchmark XT automated slide stainer (Ventana Medical Systems, Tucson, AZ, USA), a machine utilized in clinical diagnostics. Standard procedure for automated staining included staining positive and negative controls. Consequently, this was performed during PR staining.

5.1.5 ISH

ISH provides insight into both the expression levels of miRNAs and the cellular localization[187], which was of outmost importance in this thesis. Methodological challenges include the same pre-analytical challenges as with IHC, elucidated in chapter 5.1.3. miRNAs have several characteristics that challenges ISH detection: 1) small size, 2) similar sequences between miRNAs and 3) tissue-specific expression[188]. These characteristics entails the demand for highly sensitive detection methods. This is ensured by Locked Nucleic Acid (LNA) modification of the miRNA probes, increasing detection sensitivity. It provides an increased base paring affinity between probe and miRNA[187]. Additional ISH optimization to ensure reliable results was performed, as elaborated in chapter 3.1.4.1.

5.1.6 Staining evaluation and cutoff determination

Throughout this study, we used a semi-quantitative scoring approach when determining IHC and ISH biomarker staining level. This is an easy and accessible approach. A weakness, however, is the use of a non-standardized staining evaluation protocol, challenging reproducibility and accurate comparison of results with other studies. None of the biomarkers investigated in this thesis is implemented in clinical diagnostics of NSCLC, thus a standardized scoring protocol is yet to be established. All scoring was performed manually by the use of light microscopy, except for ERα where the ARIOL imaging system (Applied Imaging Corp., San Jose, CA, USA) was used. This is time-consuming work, and inter-observer variability between scorers may occur. This is attempted minimized with measures elaborated in chapter 3.1.5. Avoiding inter-observer variability is possible by automated quantification. Imaging software is now able to separate staining in different tissue compartments, facilitating quantification of biomarker expression in cancer cells as well as stromal cells, which has been key throughout this thesis[189]. Automated digital image analysis (DIA) provides an objective and efficient biomarker characterization, minimizing the potential of inter-laboratory and inter-observer discrepancy[190]. Automated quantification is continuously implemented in our research group, and will provide a more efficient staining evaluation.

We have chosen to dichotomize all our biomarkers in high/low or positive/negative expression according to the mean value (PR, ERα, ERβ, AR, miR-143) or zero (miR-145). Both zero and mean value are commonly used cutoffs making it more accessible in terms of comparison with similar studies. Further, using the mean value minimizes the risk of type I errors (false positive expression). However, it may not represent the biological relevant cutoff, thus increasing the chance of type II errors (false negative). For miR-145, zero was the cut point that best differentiated between patient groups, subsequently this was the chosen

cutoff. As our study is hypothesis generating, choosing biomarker cutoff based on a minimal p-value approach, as performed for miR-145, is appropriate. This approach may also contribute to discovering small patient groups that may benefit from a certain treatment, as is the case for NSCLC adenocarcinoma patients with activating EGFR mutations. They constitute only 10-16% of NSCLC adenocarcinoma cases in Europe, but have shown significantly improved disease-free survival when treated with EGFR-TKI[191].

5.2 Discussion of results

A major strength of the studies presented in this thesis is the large, unselected cohort of NSCLC patients. We report a long follow-up of our patient cohort, further strengthening our results.

5.2.1 Discussion Paper I

Our first paper elucidates the prognostic role of PR in NSCLC. We revealed a positive prognostic impact of high PR expression in the tumor surrounding stroma. Interestingly, PR expression in tumor epithelial cells emerged as an independent negative prognosticator only in the female patient group.

Reports on PR prevalence and its prognostic significance in NSCLC have been somewhat conflicting. Some groups report a high PR prevalence[122,192], while others report low or no expression in NSCLC[193,194]. In our cohort, we found PR expression both in epithelial tumor cells and in tumor surrounding stromal cells (for details on expression levels and cell type/localization, see **Table 2**, **paper I**). We used a reliable antibody, routinely used in clinical diagnostics for detecting PR expression.

To our knowledge, we are the first group to report diverse PR prognostic effects related to its localization, in epithelial tumor cells versus tumor surrounding stroma. Tissue specific effects

of progesterone signaling have been previously reported in other malignancies: Growth-promoting PR-signaling reported in breast cancer[70], astrocytoma[195] and osteosarcoma[196], whereas PR-mediated growth inhibition has been reported in uterine cancer[197]. Further, Kim et al.[197] described a compartment specific function of PR in uterine cancer. They observed a pivotal role of PR in endometrial stromal cells, indicating that the stromal PR expression is responsible for the inhibition of endometrial epithelial cells. This inhibiting effect was explained by blocking of estradiol-induced mitogenic mediators[197]. Little is known regarding PR signaling in NSCLC cells, and even less is known with respect to PR signaling in the stromal compartment. Our observation of PR as a positive prognosticator when expressed in stromal cells may indicate a similar endometrial-like function in NSCLC, as observed by Kim et al.[197]. The inhibition of epithelial cells may be explained by a stromal-epithelial cross-talk mediated by PR signaling.

PR was recently confirmed as a positive prognosticator in a small cohort of lung adenocarcinomas[198]. Several groups have shown that a loss of PR expression is associated with a worse outcome in cancer, including lung cancer[120,199,200]. This has also been observed in endometrial cancer, where poorly differentiated and more aggressive tumors usually show a lower degree, or even a total loss of PR expression[197].

Functional studies investigating the role of PR in tumor surrounding stromal cells in NSCLC are needed to fully comprehend their complex signaling pathways. Additionally, it is important to consider that PR signaling in the lung may be ligand-independently, as observed in breast cancer cells[201].

In our NSCLC cohort, PR expression in tumor epithelial cells was found to be a negative prognosticator in the female patient group[152]. An explanation for this may be found in breast cancer cells. Here, PRs are expressed primarily in tumor epithelial cells, and contribute to breast cancer cell proliferation through a non-genomic signaling[197].

Most cases of NSCLC occur in elderly men and postmenopausal women, where serum progesterone levels are negligible. Autocrine production of progesterone in NSCLC cells has, however, been detected and found to correlate with PR expression and progesterone-mediated signaling[122,126].

An increase in lung cancer mortality is reported in postmenopausal women receiving HRT treatment composed of estrogen and progestin. This is not observed in female patients taking estrogen-only HRT[23,202]. Further, cell line studies have reported a progestin mediated vascular endothelial growth factor (VEGF) secretion by NSCLC cells, thus indicating local angiogenesis and tumor growth [121]. We found a correlation between PR expression and the angiogenic marker platelet-derived growth factor C (PDGF-C), indicating that progesterone mediated angiogenesis in NSCLC may be executed through cross-talk signaling with PDGF-C. The aforementioned reports combined with our results, indicate an association between tumor promoting qualities and PR expression and signaling.

5.2.2 Discussion Paper II

Herein, we showed that high tumor cell AR expression was a negative prognosticator in all patients, while nuclear tumor ERβ expression emerged as a negative prognosticator in female patients.

High aromatase (AR) expression has been associated with an adverse prognosis in NSCLC patients, which is in concordance with our findings[203,204]. As is seen in breast cancer, lung cancer cells appear to produce large amounts of estrogens locally, by an aromatase-mediated conversion of androstenedione to estradiol[135,153,203]. In our NSCLC cohort, high ERβ tumor expression emerged as an independent negative prognosticator in female patients. This is in agreement with several reports on the prognostic role of ERβ in NSCLC[120,131,136]. Gender-specific survival discrepancies have previously been described for ERβ expression in NSCLC. Schwartz et al.[205] reported a favorable prognosis in male patients with a high ERβ

expression, while Stabile et al.[120], found a high ERβ expression to correlate with an inferior outcome.

The associations between a high AR and high ERβ expression with an unfavorable prognosis shown in our study, may be explained by the mitogenic qualities following the activation of the ER-pathway in NSCLC, as described in chapter 1.2.1 and 1.3.2. Further, we found interesting correlations between our investigated hormonal biomarkers and important angiogenic growth related factors including VEGFR and PDGFA[153]. Estradiol has shown to induce VEGF secretion and also to increase the number of cancer stem cells, self-renewing cells with high malignant capacity, in NSCLC[121]. This may provide further explanations for the negative prognosis accompanying high ERβ or AR expression.

The improved survival following implementation of hormone therapy in breast cancer treatment, renders hope of a similar breakthrough in lung cancer therapy. An overview of important milestones in elucidating hormone-targeted therapy in NSCLC is presented in chapter 1.3.2, **Table 2**. In preclinical studies, treatment with E2 has shown to promote the proliferation and progression of lung cancer cell lines *in vitro* and lung tumor xenografts *in vivo*[133,206-208]. The estrogen-mediated effects in NSCLC was inhibited by fulvestrant, a renowned ER antagonist[128]. Further, in a Canadian study by Lother et al.[209], antiestrogen exposure in 156 of 2320 female NSCLC patients, was associated with improved survival. A recent report by Hamilton et al.[86] demonstrated that blocking of estrogen signaling by fulvestrant augmented lung carcinoma cells susceptibility to immune-mediated lysis. These reports provide a rationale for targeting the estrogenic pathway in NSCLC treatment, and the latter report, emphasize the potential of combining estrogen-targeted treatment with immune-mediated therapies. As hormonal therapy targeting the estrogenic pathway has been used in clinical practice for some time now, they are widely available and their low toxicity profile is well-established[127].

Preclinical studies on aromatase inhibitors in NSCLC have also shown to prevent tumor cell growth[132,210]. Weinberg et al.[210] early proposed AR as a therapeutic target in NSCLC by demonstrating inhibition of lung tumor growth *in vitro*, when treating tumor cells with the aromatase inhibitor anastrozole. A phase II study evaluating a multi-targeted therapy of advanced NSCLC with the aromatase inhibitor letrozole and the mammalian target of rapamycin (mTOR) inhibitor everolimus, was, however, terminated due to serious adverse events[211]. Recently, joint treatment with aromatase inhibitors and NSAIDs was proposed as an NSCLC prevention in smokers. The combined treatment caused a reduction in circulating E2 and inhibited recruitment of macrophages and pro-inflammatory cytokines prior to carcinogen exposure (tobacco smoke)[212].

EGFR is an important therapeutic target in NSCLC, but resistance towards EGFR-TKIs represents an emerging challenge[213]. ER- and EGFR-signaling share several common downstream mechanisms, and a reciprocal expression regulation is shown for the biomarkers[214]. Recent publications indicate ERβ expression to be associated with the development of EGFR-TKI resistance, and demonstrates that treatment with an ERβ inhibitor sensitizes NSCLC cells to EGFR-TKIs[213,214]. These findings provided a treatment approach with combining an EGFR-TKI with an ER-antagonist in overcoming the challenges of EGFR-TKI resistance[214]. Similarly, ERβ was recently shown to increase the sensitivity of NSCLC cells to chemotherapeutic agents, further substantiating their potential as complementary therapeutic targets[135,215].

5.2.3 Discussion Paper III

Herein, we present stromal expression of miR-143 and miR-145 as positive prognosticators in female and male NSCLC patients, respectively.

The positive prognostic impact of high stromal miR-145 expression in our NSCLC cohort, has previously been indicated by research groups reporting a significant correlation between

a low miR-145 expression and poor outcome in NSCLC and prostate cancer[216,217]. Contradicting results have, however, been published for bladder cancer and esophageal cancer, indicating that high expressions of miR-143 and miR-145 are associated with an unfavorable prognosis[218,219]. This may imply a tissue-specific role for miR-143 and miR-145 expression.

The miRNA-cluster 143/145 is considered a tumor-suppressor. This is concordant with our observations in cell line studies and with observations made by other groups, reporting the down-regulation of the miRNA-cluster 143/145 in several malignancies, including breast, colon, prostate and lung[145,146]. Nevertheless, by the use of ISH, we observed an upregulation of the miR-143/145 expression in tumor tissue (tumor epithelial cells and tumor surrounding stroma) resected from our NSCLC cohort. It is becoming increasingly evident that the down-regulation of this miR-cluster is a dynamic process, and that the heterogeneity of human tumors challenges a precise expression profiling of the aforementioned miRNAs. This may explain the discrepancy in expression levels reported in various malignancies and different cellular compartments[220,221].

Deregulation of the miRNA-143/145 is reported as an early event in cancer progression, and is associated with a poor prognosis (lymph node metastasis and with an advanced stage of disease)[145]. Our cell line studies showed that transfection of NSCLC cell lines with miR-143 and/or miR-145 primarily mediated a decrease in proliferative and migration capacity. This is in concordance with others, and substantiate the tumor-suppressive effects of this miRNA cluster[222,223]. Recent results are, however, questioning the tumor suppressor function of this miRNA-cluster as publications reporting tumor promoting qualities are gaining ground[145,224,225]. There is an ongoing debate on the role of these miRNAs in the NSCLC tumor microenvironment, as there are mounting evidence that the miRNAs mainly exert their function by affecting the stromal cells, and not the epithelial cells[225]. This highlights the

importance of considering the tumor stroma when investigating the expression and prognostic relevance of biomarkers, as in this thesis. Much remains to be elucidated regarding the biological function of miR-143 and miR-145 in NSCLC including their positive prognostic role, when highly expressed in the stromal compartment.

The miRs are associated with a wide variety of genes by regulating their expression post-transcriptionally, and it may appear that their functions are not only cell type-specific, but also gender-specific, as previously proposed for a subset of miRNAs[226]. This may explain our results indicating a positive prognostic role for stromal miR-143 expression in female patients, and a positive prognostic role for stromal miR-145 expression in male patients.

We found correlations between the investigated miRNAs and our previously investigated SHRs, indicating a feasible interaction between the aforementioned miRNAs and SHRs, as already demonstrated in breast cancer tissue[227]. Further, miR-145 has been shown to deregulate ERα expression and interact with local estradiol synthesis in the breast[148]. We report a highly significant correlation between AR and the aforementioned miRNAs, indicating that the same mechanisms may be present in the lung. This may further explain the gender-related impacts observed herein.

The ability to introduce exogenous miRNAs by "miRNA replacement therapy" is a promising novel treatment opportunity[221]. Further, miRNAs may be used in multi-targeted treatment with current treatment options to prevent the development of drug resistance[228,229]. The very first miRNA replacement therapy in the clinical trial, MesomiR-1, recently completed phase 1. This new treatment approach was performed in thoracic cancers, based on the miR-15/107 group of miRNAs, and preliminary results are promising[144]. However, a precise miRNA expression and effect profile for every tumor and tumor microenvironment is inevitable prior to implementing this as an established treatment regimen[221].

6 CONCLUSIONS

The revealing of reliable biomarkers as prognostic and predictive molecular proteins and aiming to improve cancer treatment and survival, has been the ambition of cancer research groups in the recent decades. Finding biomarkers that may stratify patients within different treatment subgroups is essential for individual targeted cancer treatment. It is however important to keep in mind the genetic instability in the hallmarks of cancer, potentially challenging the applicability of candidate prognostic markers. Tumor heterogeneity represents an additional challenge in the study if potential biomarkers. Discrepant expression and prognostic relevance according to tumor compartment is presented in all three studies comprising this thesis, substantiating the disparities in compartment specific features.

The discovery of novel biomarkers in NSCLC, including EGFR and ALK, have shown an improve in lung cancer survival. There is, however, a constant need for further stratification of this heterogeneous patient group. Only 15-18% of NSCLC patients survive beyond 5 years, and the need for reliable prognostic and predictive biomarkers is pivotal. Stimulated by other research groups investigating the prognostic role of biomarkers known to be relevant in hormone-related malignancies such as in breast, ovary, uterus and prostate, we aimed to elucidate a possible role for endocrinology-related markers in lung cancer. This renders the possibility for discovering new driver molecules in malignancies previously thought not to be influenced by gender-related hormones and hormone receptors as is the case in NSCLC. Pioneer work in NSCLC during early 2000s, proposed a prognostic role of SHRs, and suggested their potential role as predictive markers in NSCLC.

Herein, we present three studies of endocrinology-related biomarkers in NSCLC, with emphasis on the female sex steroid hormone receptors. By the use of acknowledged laboratory techniques, including IHC, ISH and functional cell line studies, we performed a

thorough and comprehensive expression profiling of these hormone receptors and associated miRNAs on our NSCLC cohort using TMA as an efficient investigation method.

In the first study, we did not only confirm the presence of PR in cancer and stromal cells in NSCLC, but we further emphasized PRs pivotal role in NSCLC tumorigenesis by demonstrating divergent, independent impacts on survival when expressed in different cellular compartments. Stromal PR expression emerged as an independent positive prognosticator, while tumor epithelial PR expression was an independent negative prognosticator in female patients. Herein, we revealed a worthwhile opposing compartment-related impact on survival. Thereby, demonstrating the importance of considering the stromal compartments role in tumorigenesis when elucidating the potential prognostic relevance of a biomarker. Due to the discrepant PR signaling observed and reports indicating HRT treatment with estrogen plus progestin is associated with an increase in lung cancer death, it will be important to consider the risk-benefit for women with an increased lung cancer risk who are considering combination HRT therapy.

In the second paper, we confirmed that AR and ERβ are associated with an unfavorable outcome in NSCLC, where ERβ emerged as a significant prognosticator exclusively in female patients. Consequently, as for PR, we revealed gender-related impacts according to biomarker expression in NSCLC, further substantiating results indicating NSCLC is a disease with apparent differences between male and female patients. Female gender is already an established prognostic factor in NSCLC, furthermore certain biomarkers may be candidate prognosticators and probably predictive markers solely for female NSCLC patients. This may represent a milestone for more individualized and hormonal-related treatment of lung cancer. However, validation in prospective trials, as well as standardized detection method and staining interpretation, is of course necessary prior to such an implementation.

In our third paper, we showed positive prognostic impacts of stromal miR-143 and miR-145 expression in a gender-related manner. We strengthened our tissue-based findings with functional cell line studies. This provided a unique possibility to explore the pathophysiology of NSCLC, and elucidate miRNAs role in tumorigenesis. Further, we revealed correlations with SHRs indicating hormone-related signaling may be involved in the gender-specific survival benefit observed. We report discrepant results on biomarker expression in our tumor tissue versus our cell lines, indicating specific mapping of miRNA expression levels in cancer cells may pose a challenge. miRNA replacement therapy may represent a solution to the development of treatment resistance in NSCLC, due to the multitude of target genes regulated by miRNas.

This thesis is a contribution to elucidating the myriad of tumor cell signaling causing disease, relapse, metastasis and eventually death for NSCLC patients. We hope our results may be implemented in future stratification of this patient group, as prognostic or predictive markers. Supplementary studies on endocrinology-related biomarkers in relation to gender, age and hormone-associated gene mutations may pose an interesting field for further research.

Even though no prognostic biomarkers are affirmed in NSCLC, ongoing research may eventually find the missing link that can aid stratification, and hopefully survival, in this patient group. This work and further investigations may be inspired by the words of Elon Musk;

"When something is important enough, you do it even if the odds are not in your favor"

Elon Musk

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PAPER I

PAPER II

PAPER III