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Tumor hypoxia and hypoxic markers in non-small cell lung cancer

An experimental in vitro study and a retrospective cohort study

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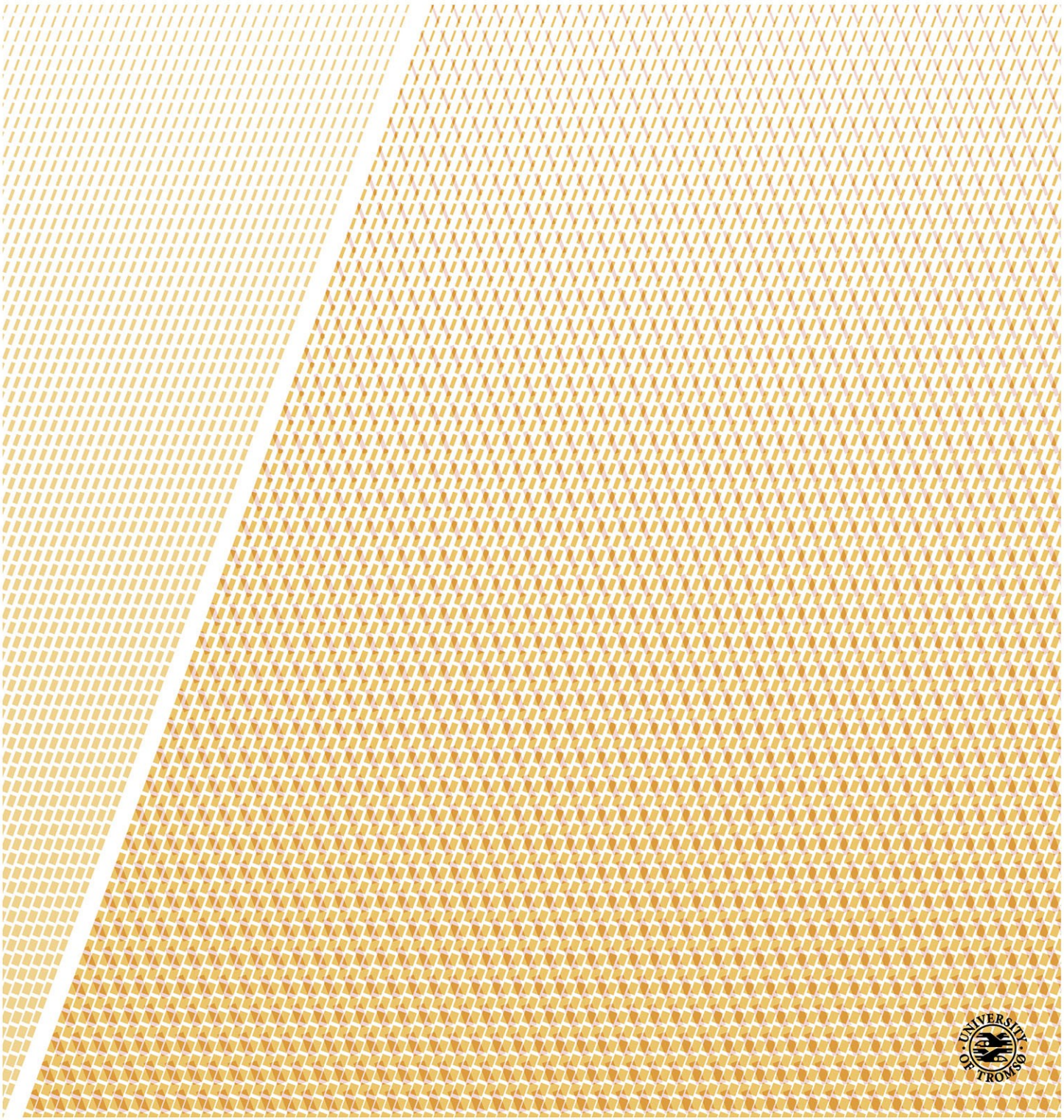

CONTENTS

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LIST OF PAPERS

The thesis is based on the following papers:

I. Diverging prognostic impacts of hypoxic markers according to NSCLC histology

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II. In NSCLC, VEGF-A response to hypoxia may differ between squamous cell and adenocarcinoma histology

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III. Monocarboxylate transporters 1-4 in NSCLC: MCT1 is an independent prognostic marker for survival

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IV. Positive prognostic impact of miR-210 in non-small cell lung cancer

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LIST OF ABBREVIATIONS

AC - Adenocarcinoma

ALK - Anaplastic lymphoma kinase

ATP - Adenosine triphosphate

BAC - Bronchioloalveolar carcinoma

CAFs - Carcinoma-associated fibroblasts

CAIX - Carbonic anhydrase IX

CT - Computed tomography

DAB - 3-3'diaminobenzidine

DSS - Disease-specific survival

EBUS - Endobronchial ultrasound

EGFR - Epidermal growth factor receptor

ELISA - Enzyme-linked immunosorbent assay

EUS - Esophageal ultrasound

FGF-2 - Basic fibroblast growth factor-2

GLUT1 - Glucose transporter 1

HIER - Heat-induced epitope retrieval

HIF - Hypoxia-inducible factor

HRP - Horseradish peroxidase

IHC - Immunohistochemistry

IL-1β - Interleukin 1β

IL-6 - Interleukin 6

ISH - *In situ* hybridization

LCC - Large cell carcinoma

LDH - Lactate dehydrogenase

LNA - Locked Nucleic Acid

MCT - Monocarboxylate transporter

miRNA - MicroRNA

MRI - Magnetic resonance imaging

MSCs - Mesenchymal stem cells

MVD - Mean vascular density

NADH - Nicotinamide Adenine Dinucleotide plus Hydrogen

NSCLC - Non-small cell lung cancer

PDGF-BB - Platelet-derived growth factor BB

PET - Positron emission tomography

RT-PCR - Real-time polymerase chain reaction

SCC - Squamous cell carcinoma

SCLC - Small cell lung cancer

TGF-β - Transforming growth factor β

TMA - Tissue microarray

VEGF - Vascular endothelial growth factor

VEGFR - Vascular endothelial growth factor receptor

1. INTRODUCTION

An important progress in the treatment of advanced non-small cell lung cancer (NSCLC) is the identification of driver mutations of cancer growth, and the subsequent development of agents that target these genetic alterations. However, heterogeneity in driver mutations is a hallmark of NSCLC, and a key reason why NSCLC patients that share the same histology and clinical stage can have different clinical outcomes and responses to treatment. Hence, combining both molecular and clinical information offers better means of customizing treatment for the individual NSCLC patient. Furthermore, the identification of subgroups of patients with targetable driver mutations have led to an improved patient outcome, together with an intense and ongoing research to identify new significant biomarkers and targeted therapies that can be used for other subgroups of patients with NSCLC.

Genetic alterations in the cancer cells are considered to drive tumor growth and progression. Furthermore, the microenvironment of the cancer cell is known to influence the cancer phenotype. Therefore, when studying the prognostic impact of molecular markers, not only the expression in cancer cells must be evaluated. Additionally, the prognostic impact of the same biomarkers in the surrounding microenvironment must be taken into the account, since the microenvironment often assists cancer cells in their growth, infiltration and metastasis. Besides, targeted therapies may also affect these cells of the tumor environment.

The main aim of this thesis was to investigate the prognostic impact of hypoxia-related markers and their relative expression in NSCLC. *In vitro* cell lines representing the two major subtypes and carcinoma-associated fibroblasts of NSCLC and a retrospectively collected cohort of 335 patients diagnosed with NSCLC stage I-IIIA were used in the thesis.

2. BACKGROUND

2.1 Lung Cancer

2.1.1 Epidemiology

Globally, lung cancer is the major contributor to cancer mortality, accounting for 1.4 million deaths in 2008[1]. In Norway, lung cancer is the third most common cancer in both men and women, with 1618 and 1224 new cases respectively in 2011[2]. In terms of mortality numbers, also in Norway, lung cancer is the major cause of cancer-related deaths in both sexes, responsible for 1267 and 917 deaths in men and women in 2011 (Figure 1).

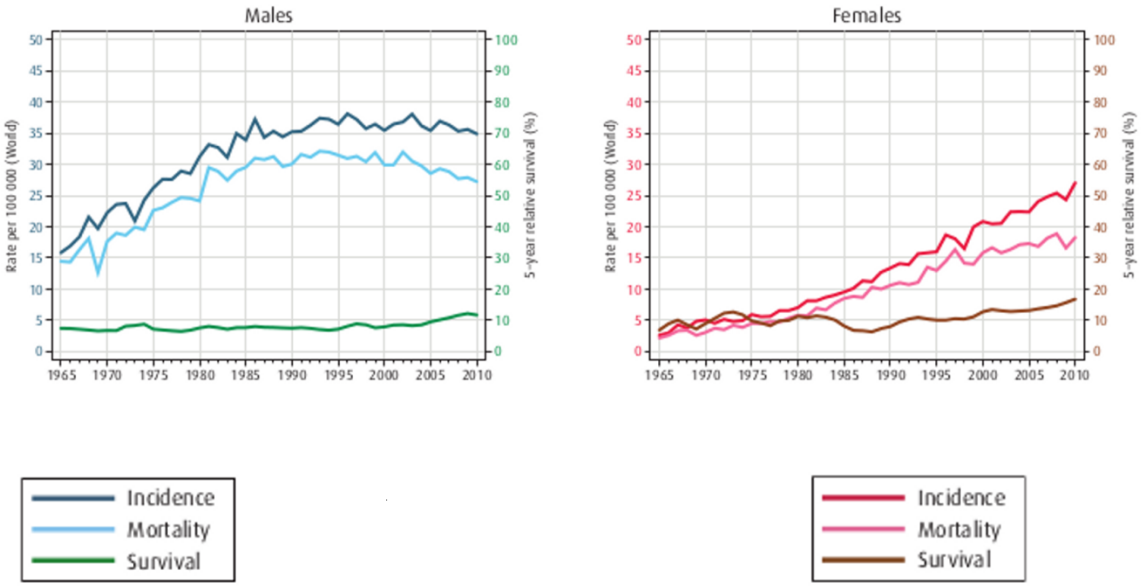

Figure 1: Lung cancer trends in Norway (1965-2010). Incidence and mortality rates and 5-year relative survival proportions (adapted from[2]).

With incidence and mortality rates being similar, poor survival is still a trait of lung cancer. However, the estimated relative lung cancer survival in Norway has had a modest upturn from the period 2002-2006 to 2007-2011, with an improvement from 13 to 17 per cent in women and 9 to 12 per cent for men[2]. These changes might reflect earlier detection, a genuine improvement of lung cancer management, less co-morbidity or changes in other factors that contribute to improved life expectancy[3]. Smoking is the major cause of lung cancer, making the disease largely preventable. Smoking accounts for 80% of the lung cancer incidences in men and at least 50% of the lung cancer cases in women[1]. The lung cancer risk increases with the number of daily cigarettes smoked and number of years smoking[4]. Passive smoking is also a risk factor[5]. In Norway, and globally, lung cancer incidence trends between genders largely reflects the phases of the smoking prevalence in men and women 20 years earlier[1] (Figure 2).

Approximately 25% of lung cancer patients worldwide are never-smokers[6]. Other known risk factors for lung cancer are exposure to carcinogens such as asbestos, radon, arsenic and polycyclic aromatic hydrocarbons[1]. Never-smokers with lung cancer have been suggested a separate entity due to differences in epidemiological, molecular and clinical characteristics[6]. For example, never-smokers with lung cancer are reported to respond better to chemotherapy compared to lung cancer patients who are former or current smokers[7]. Furthermore, never-smokers with primary adenocarcinoma (AC) of the lung are shown to live longer compared to former or current smokers[8].

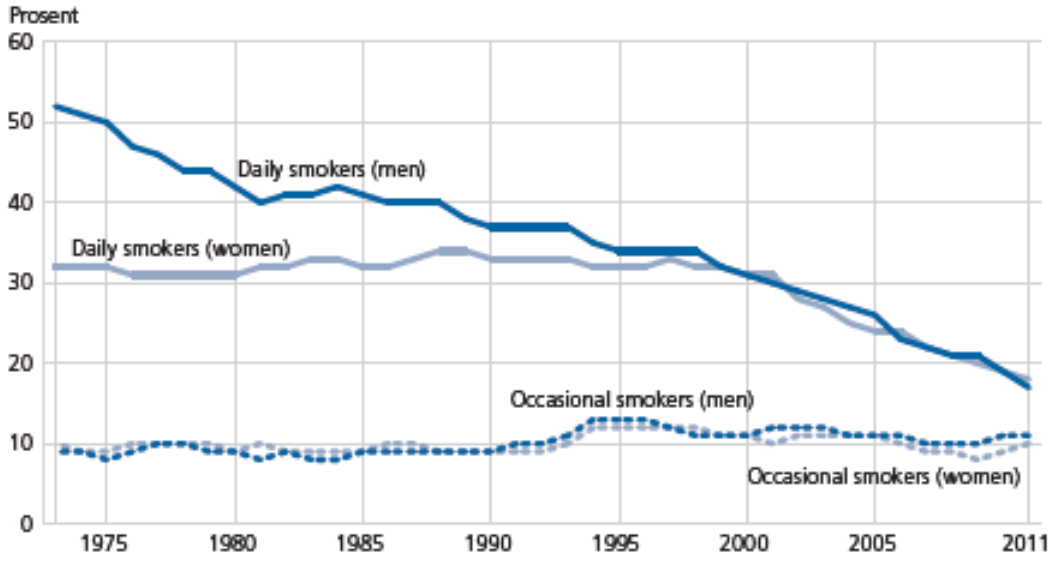

Figure 2: Proportions of daily and occasional smokers in Norway, by sex (16-74 year old) (Figure adapted from[9]).

2.1.2 Histopathology

For clinico-pathological reasons, lung cancer is divided in two main categories: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC)[6]. Eighty-five per cent of lung cancer patients in Norway are diagnosed with NSCLC. This subgroup is further divided into three main histological subcategories: squamous cell carcinoma (SCC), AC and large cell carcinoma (LCC)[6].

A major global and national trend is a decrease in SCC and a sharp rise in AC during the last decades[10]. AC has become the most prevalent histologic subtype of lung cancer, probably due to the increasing use of filter cigarettes with lowered nicotine and tar content[6]. AC is also the most frequent histologic subtype among never-smokers[6]. A

new classification of lung AC was released in 2011[11]. One of the key differences from the former classification from 2004 is that the previous term bronchioloalveolar carcinoma (BAC) is included in the AC. This AC is divided into pre-invasive lesions (atypical hyperplasia or *in situ* lesions), minimal invasive AC and invasive AC. The invasive AC is further divided into subgroups according to their growth patterns, like lepidic, acinar, papillary, micropapillary, colloid, fetal and enteric. These changes have little practical influence on the results of this thesis, as the BACs have been included in the AC subgroup for the statistical calculations.

In recent years, therapeutic decisions in advanced NSCLC have become more dependent on histological diagnosis[12]. For instance, bevacizumab and pemetrexed is restricted to treatment of patients with non-SCC only[13]. For the antiangiogenic agent bevacizumab, the restriction is due to life threatening hemorrhage after treatment of patients with SCCs[13]. For pemetrexed, patients with non-SCC appear to have the greatest benefit from treatment with this chemotherapeutic agent[13]. Further, as epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase (ALK) rearrangements are almost solely seen in ACs, treatment with erlotinib and crizotinib respectively, is nearly exclusively administered to patients with ACs[11,14,15].

2.1.3 Diagnosis, staging (TNM) and prognosis

Sadly, as much as 70% of lung cancer patients have advanced stages at time of diagnosis[16]. This is due to few and vague symptoms at the early phase of the disease[17]. The symptoms are dependent on the localization of the primary tumor and the metastases[17]. Common symptoms of primary lung cancer are cough, dyspnea, chest pain and hemoptysis[17]. At advanced stages of the disease the common symptoms are reduced appetite, weight loss and fatigue[17].

Today, there is no lung cancer screening program in Norway. Low dose helical CT (computed tomography) screening of high-risk patients (≥30 pack years) has been observed to reduce lung cancer mortality[18]. However, before a screening program can be implemented in the clinic, further data from ongoing screening studies, an improved selection of high-risk groups, improved algoritms for dealing with mostly false positive findings and adequate CT capacities are needed[18].

When lung cancer is suspected, the patient undergoes chest X-ray and CT scan of the chest (including the supraclavicular fossa) and upper abdomen (including the liver and the adrenal glands)[19]. If a lung tumor is detected, biopsies/cytology specimens have normally been obtained by bronchoscopy or for peripheral tumors by CT/ultrasound-guided transthoracic core needle biopsy/fine needle aspiration[19]. Today, PET (positron emission tomography), esophageal ultrasound (EUS) and/or endobronchial ultrasound (EBUS) has been added to the staging workup[19]. To rule out possible brain metastases, especially in the case of SCLC, brain MRI (magnetic resonance imaging) is often performed during staging procedures[19].

In 2007, IASLC published a new TNM classification for lung cancer[20] (Table 1). In general, the TNM classification is essential to separate patients in different risk groups and to select the appropriate treatment regime.

Table 1. The seventh edition of TNM classifications and stage groupings (Table adapted from[20])

Stage	Sub-stage	T Category	N Category	M Category	5-year survival
Occult carcinoma		Tx Primary tumor not assessed or proven only by cells	N0 No regional lymph node metastasis	M0 (no distant metastasis)	
Stage 0		Tis carcinoma in situ	N0	M0	
Stage I	**IA**	T1a Tumor ≤ 2 cm	N0	M0	73%
		T1b Tumor ≤ 3 cm >2 cm			
	IB	T2a Tumor ≤ 5cm > 3 cm	N0	M0	58%
Stage II	**IIA**	T1a	N1 metastasis in ipsilateral hilar LN	M0	46%
		T1b	N1		
		T2a	N1		
		T2b Tumor ≤ 7 cm > 5 cm	N0		
	IIB	T2b	N1	M0	36%
		T3 Tumor > 7 cm/invading chest wall, pleura or pericardium/in the main bronchus <2 cm from carina	N0		
Stage III	**IIIA**	T1	N2 metastasis in ipsilateral mediastinal and/or subcarinal lymph nodes	M0	24%
		T2	N2		
		T3	N1		
		T3	N2		
		T4 Tumor invading mediastinum, heart, great vessels, trachea, esophagus, vertebral body, carina or tumor in another ipsilateral lobe	N0		
		T4	N1		
	IIIB	T4	N2	M0	9%
		Any T	N3 metastasis in contralateral mediastinal, hilar, scalene or supraclavicular LN		
Stage IV	**IV**	Any T	Any N	M1A pleural or pericardial effusion or separate tumor in contralateral lobe M1B distant metastasis	13%

T, N and M sub classification description appears only once

2.1.4 Treatment of NSCLC

2.1.4.1 Limited stage/curable NSCLC

For patients with stage I NSCLC, surgery is the treatment of choice and no adjuvant or neoadjuvant treatment is given[19]. Patients with stage II and stage IIIA NSCLC, surgery and adjuvant chemotherapy are the treatment regimen of choice[19,21]. The preferred adjuvant regimen is four cycles of cisplatin and vinorelbine[19]. In addition, concurrent or sequential thoracic radiation is given to stage IIIA pN2 patients[19,22-24]. Postoperative radiotherapy is indicated for patients where positive surgical margins are detected and/or for patients with pN2 or pN3 disease[19,25]. If the patient is not technically or medically fit for surgery, radiation (66-70 Gy) with curative intention is given, with or without chemotherapy[19].

2.1.4.2 Advanced NSCLC

Chemotherapy, radiation therapy or targeted therapy with a palliative goal is the main treatment regimen available for patients with advanced NSCLC[19]. For some of the patients in this group, no therapy is given due to severely reduced performance status and the seriousness of their disease.

Standard treatment of advanced NSCLC is platinum doublets; platinum (carboplatin or cisplatin) in combination with docetaxel, gemcitabine, vinorelbine or pemetrexed[19,26]. Platinum-doublet chemotherapy is administered as 3-4 cycles[19]. In Norway, carboplatin and vinorelbine is the platinum-doublet combination of choice based on efficacy and toxicity profiles[19,27]. Some reports are implying that maintenance

chemotherapy with pemetrexed may have effect in ACs, and clinical practice guidelines recommend maintenance chemotherapy in selected patients[19,28].

Palliative thoracic radiotherapy is recommended to patients with advanced NSCLC and symptoms from the central airways[19,29,30]. For patients with brain metastasis or painful chest wall/bone metastasis, palliative radiotherapy should be considered[19,31-33].

In a cohort of 240 Norwegian lung cancer patients selected for surgery, 7,5% had EGFR-activating mutation (Mut+)[34]. Since therapies targeting the EGFR tyrosine kinase mutations show dramatically increased progression-free survival in the Mut+ subgroup of lung cancer patients[35-38], all patients with NSCLC in Norway are recommended for EGFR-activating mutation testing[19]. If EGFR Mut+ is confirmed, tyrosine kinase inhibitors (erlotinib/gefitinib) is recommended as first line treatment, instead of chemotherapy[19].

The monoclonal antibody bevacizumab have shown clinical benefit in treatment of metastatic colorectal cancer[39]. In non-SCC NSCLC, Sandler and co-workers observed a 2 month survival benefit[40]. However, the follow-up European randomized phase III trial on bevacizumab in non-SCC did not demonstrate any survival benefit by adding bevacizumab to chemotherapy[41]. Consequently, bevacizumab is not recommended in our national lung cancer treatment guidelines[19].

2.2 Tumor microenvironment

The previous view that cancer cells in solid tumors act independently of cells in the surrounding tissue has changed dramatically over the last decade[42]. Cancers are now recognized as complex tissues, with the tumor microenvironment as an important contributor to the malignant phenotype, promoting cancer initiation, growth infiltration and metastasis[42-44].

The tumor stroma constitutes all cells in the surrounding area of the tumor[45]. These include fibroblasts, myofibroblasts, inflammatory cells, mesenchymal stem cells (MSCs), adipocytes, endothelial cells, pericytes and the extra cellular matrix[45].

Cancer cells are capable of producing stroma-modulating growth factors, that disrupt normal tissue homeostasis and creates a cancer supportive microenvironment[43]. In a paracrine manner, these growth factors activate resident stromal cells, induce angiogenesis and inflammatory responses which in turn may act pro-tumorigenic[46].

Fibroblasts were intuitively considered the origin of carcinoma-associated fibroblasts (CAFs), a prominent cell type of the tumor stroma known to promote tumor growth, angiogenesis, inflammation and metastasis[45]. But with the growing interest in and studies on CAFs, various origins have subsequently been proposed including normal fibroblasts, MSCs, endothelial cells and *trans*-differentiated epithelial cells[45]. Recently, due to the many controversies around CAFs, including its cell of origin, a new definition was put forward leaving the view that CAFs is a distinct cell type[45]. In the new definition, cells in the tumor stroma with traits like mesenchymal appearance and tumor-promoting ability, should be considered to be in a "CAF state"[45]. The new definition

implies that cells of different origins can exhibit a "CAF state". Stromal cells in a "CAF state" are promising targets, since "CAF state" cells enable many of the hallmarks of cancer *and* are known to modulate the cancer cells sensitivity and exposure to anti-cancer drugs[43].

The immune cells of the tumor microenvironment comprise both innate (macrophages, neutrophils, dendritic cells, myeloid-derived suppressor cells and natural killer cells) and adaptive immune cells (B and T lymphocytes)[47]. The immune cells are recruited to tumors in various ways, ranging from oncogenic signaling that trigger transcription of tumor-promoting cytokines and chemokines to necrotic cell death (due to hypoxia or cancer therapy) in the tumor with release of pro-inflammatory factors[47]. Like "CAF state" cells, the immune cells communicate with cancer cells (and stromal cells) by means of direct contact or chemokine and cytokine production potentially impacting every step of tumor progression, from initiation to metastasis[47]. During tumorigenesis, it is assumed that anti-tumor immunity and tumor-promoting inflammation co-exist[47]. What decides in which direction the balance is tipped is the expression of various factors as well as the activation state and abundance of various immune cells in the tumor microenvironment[47]. Hence, immune cells of the tumor microenvironment may represent target opportunities for cancer therapy and prevention[47].

For development of future NSCLC therapies, understanding the nature of the tumor microenvironment may be equally important as understanding the nature of the cancer cells[44]. Consequently, our research group has sampled NSCLC tumor stroma, in

addition to NSCLC cancer cells, to be able to study the expression and prognostic impact of hypoxia-related markers and the angiogenic response to hypoxia. Further, it has been stated that the profile of molecular factors produced in the tumor microenvironment may be more important to study than the cellular origin of these molecular factors[47]. Therefore, when using the tissue microarray (TMA) platform, we chose to evaluate the hypoxia-related marker expression in the tumor stroma as a whole.

2.3 Hypoxia

2.3.1 Hypoxia and hypoxia response pathway regulation

Hypoxia (inadequate oxygen levels, usually defined at ≤2% O_2) develops in solid tumors due to uncontrolled growth of cancer cells and insufficient angiogenesis and blood flow[48,49]. Intraoperative measurements of oxygen tension in patients with NSCLC tumors have shown that hypoxia is a feature of NSCLC[50]. Tumor hypoxia has been associated with a more malignant tumor phenotype, with reduced sensitivity to chemotherapy and radiotherapy in several cancers[48,51]. This has stimulated intensive research into hypoxia-induced pathways and regulation of these pathways. On the other hand, hypoxia may also promote antiproliferative effect, by restricting cell proliferation, differentiation and inducing apoptosis and necrosis[52].

The primary response to hypoxia in both normal cells and cancer cells is mediated by the transcription factors hypoxia-inducible factors (HIFs)[49]. Also, recent observations link a microRNA (miRNA), miR-210, to a post-transcriptional regulation response to hypoxia in both a HIF-dependent and HIF-independent manner[53-55].

2.3.2 Hypoxia and angiogenesis

To re-establish an adequate supply of oxygen and nutrients, hypoxic tumor cells mainly through HIFs, mediate cell survival through increased expression of genes that initiate angiogenesis (the growth of new vessels from pre-existing capillaries)[56]. Angiogenesis is one of the essential alterations in cell physiology that dictate malignant growth[42]. The angiogenic activation process is termed the “angiogenic switch”, and takes place when

pro-angiogenic mediators, like vascular endothelial growth factor (VEGF)-A, out-balance anti-angiogenic mediators, like thrombospondin-1[57].

Cancer cells can gain excess to oxygen and nutrients by other mechanisms than angiogenesis, but the significance of these processes is not well understood[56]. For example, cancer cells can hijack the resident vasculature, a phenomenon called vessel co-option, or in other cases vascular mimicry occurs, in which cancer cells can line vessel walls[56]. Tumor endothelium can even be generated by cancer stem-like cells[56].

In contrast to physiologic angiogenesis, the induction of angiogenesis by transformed cells is not well organized. The blood vessels that are formed are irregular in size and shape[57]. Due to the structural malformations, chaotic blood flow, vessel leakage and increased intratumoral pressure are results from the cancer-related angiogenesis. Hence, local regions of hypoxia still prevail in malignant tumors.

2.3.3 Hypoxia, glycolysis and lactate homeostasis

Due to shifting, but prevailing areas of hypoxia, the cancer cells can no longer rely on adenosine triphosphate (ATP) generation through oxidative phosphorylation[58]. A shift in ATP generation through glycolysis by cancer cells, were identified several decades ago by Otto Warburg[58]. Glycolysis is the biochemical route where glucose is broken down to pyruvate and 2 ATPs[58]. Interestingly, glycolysis in cancer cells occurs even when there is enough O_2 to support ATP production through oxidative phosphorylation[58]. This phenomenon is termed “aerobic glycolysis” or “the Warburg effect”[58]. The most selective advantage of the Warburg phenotype is an ongoing debate. It has been

proposed that “the Warburg effect” provides a biosynthetic advantage for tumor cells, in which glycolysis allows for effective shunting of carbon to generate biomass[59]. Alternatively, it has been proposed that glycolytic metabolism arises as an adaption to hypoxic conditions during the early avascular phase of tumor development, as it allows for ATP production in the absence of oxygen[60].

The final product of glycolysis is pyruvate[58]. When O_2 is in short supply, lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactate with a simultaneous conversion of NADH (Nicotinamide Adenine Dinucleotide plus Hydrogen) to NAD^{+}[58]. Lactate is then removed from the cell through the transmembrane transporter monocarboxylate transporter (MCT) 4, to maintain intracellular lactate homeostasis[58]. Subsequently, extracellular lactate homeostasis is believed achieved by lactate uptake both by oxidative cancer cells and tumor stroma cells[61]. Alternatively, cancer cells can allocate Warburg metabolism to CAFs, exploiting their lactate production to grow in a environment low on glucose, hence symbiotically adapting with stromal cells to glucose availability[62].

2.4 Hypoxia associated molecular markers covered in this thesis

2.4.1 Paper I: HIF(1-2)αs, GLUT1, LDH5 & CAIX

HIFs are the main transcription factors that regulate cancer cells’ adaption to hypoxia[49]. HIFs are heterodimeric transcription factors, composed of an O_2-sensitive α-subunit (HIF-1α, HIF-2α, HIF-3α) and a stable β-subunit, which together binds to genes with

hypoxia responsive element sequences[49]. O_2-dependent post-translational stabilization of HIF-1α and HIF-2α subunits controls the HIF activity during hypoxia[49]. However, O_2-independent mechanisms like increased oncogenic signaling can also regulate HIFα subunits[49] .

Available evidence points to HIF-1α and HIF-2α being responsible for the majority of HIF-dependent responses to hypoxia[49]. Little is yet known about the impact of HIF-3α on tumor progression in a hypoxic environment, but in normal cells HIF-3α acts as a negative regulator of HIF-1α and HIF-2α[48,49].

The two major categories of genes regulated by HIF-1 are those genes involved in increasing O_2 delivery (e.g. VEGF) and those decreasing O_2 consumption (e.g. glycolytic enzymes)[63]. Glycolytic enzymes are involved in the enzymatic breakdown of glucose to pyruvate[64]. Pyruvate is subsequently converted to lactate by LDHs[65]. Among five isoenzymes, LDH5, also called LDHA, has the highest efficiency in catalyzing pyruvate to lactate, and is also under HIF-1 transcriptional regulation[65].

A major consequence of this metabolic switch to glycolysis is a decrease in pH caused by lactic acid production[65]. Carbonic anhydrase IX (CAIX) is upregulated in a HIF-dependent manner to export H^+ ions, and hence maintain a normal intracellular pH level[65].

Compared to oxidative phosphorylation, glycolysis produces ATP more rapidly, but it is much less efficient in terms of ATP generated per unit of glucose consumed[60]. To compensate for the low ATP yield in the glycolytic pathway, HIF-1 upregulates the expression of the glucose importer glucose transporter 1 (GLUT1)[65]

2.4.2 Paper II: VEGF-A & VEGF-C

The VEGF family expressed in mammals currently comprises five different members, with different receptor specificity and function[56]. VEGF-A (also known as VEGF) is the main component, and stimulates angiogenesis predominantly through VEGF-receptor-2 (VEGFR-2)[56].

A key regulator of VEGF-A gene expression is hypoxia and HIF-1[42,63]. In addition, VEGF-A expression is also upregulated by a variety of cytokines and growth factors, including basic fibroblast growth factor-2 (FGF-2), interleukin 1β (IL-1β), interleukin 6 (IL-6), transforming growth factor β (TGF- β), platelet-derived growth factor BB (PDGF-BB), some of which can act synergistically with hypoxia[66].

VEGF-C activates blood-vessel cells through binding VEGFR-2 and VEGFR-3[67]. VEGFR-3 is a key regulator of the formation of new lymphatic vessels (lymphangiogenesis), but has also been found to be important for angiogenesis[67]. VEGF-C expression in tumor cells may be induced by pro-inflammatory cytokines or growth factors[68]. Inflammatory cells in the tumor stroma may also be the source of VEGF-C[68].

2.4.3 Paper III: MCT 1-4

MCTs are essential for the transport of monocarboxylates such as lactate and pyruvate across cell membranes[69]. MCT1 and MCT4 have a central role in cancer cell metabolism and are critical for the metabolic communication between cells[70]. MCT1 and MCT4 are located in the cell membrane[61]. MCT1 can promote both import and export of lactate

depending on the pH gradient, while MCT4 facilitates lactate release[61,71]. MCT2 and MCT3 in cancers are less studied. MCT2 is reportedly expressed in the mitochondrial membrane, where it is involved in the import of pyruvate following lactate oxidation[72]. MCT3 exports lactate, and is reported to be expressed in retinal pigment epithelium and choroid plexus epithelium[73].

Only MCT4 is regulated by hypoxia in a HIF-1-dependent manner[74]. This is in agreement with HIF-1 responsive gene products which enhance the rates of glycolysis, and hence the need to export large amount of lactic acid is existing. The regulation of MCT1-3 remains to be fully elucidated. In fact, one study links MCT1 to p53 regulation[75].

2.4.4 Paper IV: miRNA-210

miR-210 is the miRNA predominantly induced by low oxygen tension[76]. Several studies have identified a variety of miRNAs to be induced by low oxygen tension, but the one miRNA all reports had in common was miR-210[77]. miR-210 expression is reported to be under control of HIF-1 and HIF-2, but apparently also HIF-independent mechanisms[53-55]. miR-210 regulate a vast number of genes involved in cell cycle regulation, angiogenesis, tumor growth, DNA damage repair, mitochondrial metabolism and apoptosis[78]. Consequently, miR-210 has been named “the micromanager of the hypoxia pathway”[78]. In cancer cell biology, miR-210 has been reported to be both a tumor suppressor and oncomiR. But, due to the recent discovery of miR-210, its role in cancer is still rather unresolved.

3. AIMS OF THE THESIS

In this thesis, we wanted to investigate if hypoxia-related markers were prognostic factors in NSCLC. Put differently, if they affected the pathobiology of NSCLC to such an extent that the degree of agressiveness of the cancer, and hence the patient outcome were affected. Further, we wanted to explore the angiogenic response to hypoxia in NSCLC cell lines.

More specifically the aims were:

- To investigate the prognostic significance of HIF-(1-2)αs and the HIF-regulated genes GLUT1, LDH5 and CAIX in NSCLC.
- To explore whether hypoxia induces VEGF-A and VEGF-C secretion in NSCLC cell lines and primary NSCLC cell cultures, and if the response to hypoxia is dependent on histological subtype.
- To assess MCT1-4s' prognostic impact in cancer cells and tumor stromal cells, and the potential prognostic synergetic value of metabolic interplay between tumor stromal cells and cancer cells.
- To examine the prognostic role of miR-210 in NSCLC tumor stromal and cancer cells.

4. MATERIAL AND METHODS

4.1 NSCLC cell lines

Commercial cell lines. The commercial cell lines used in the thesis were bought from ATCC (American Type Culture Collection). We chose two cell lines representing the two most common histological subtypes of NSCLC. The NCI-H520 (ATCC® HTB-182™) cell line is derived from a male patient with primary SCC of the lung. The NCI-H522 (ATCC® CRL-5810™) cell line is derived from a male patient with primary AC of the lung. Authentication certificates and validation of the cell lines were provided by the manufacturer.

Primary cell cultures. Primary cell cultures were established as an enriched population of defined cell types from freshly resected NSCLC tumor tissues. Patients who donated tissue participated after giving informed consent. The study was approved by the Regional Committee for Medical and Health Research Ethics (REK). Validated tumor samples were cut in tiny (1-1.5 mm^3) pieces. Subsequent enzymatic digestion for 1.5h in 10 ml DMEM/Ham's F-12, containing 0.8 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO, USA) was carried out. The digested NSCLC tissue was spun down, and resuspended in fresh growth medium (DMEM/Ham's F-12) supplemented with 10% FBS.

The primary AC cell line (PAC) was established after serial eliminations of CAFs. The remaining adherent tumor epithelial cell colonies were grown in a tailored serum-free medium that favors growth of epithelial cells. The epithelial cell cultures that tolerated subculturing were established as continuous cell lines. Characterization for purity and

cell identity was done by flow cytometry, using fluorescein isothiocyanat (FITC)-conjugated anti-human pan cytokeratin (Sigma-Aldrich, St. Louis, MO, USA).

The primary CAFs were characterized for cell identity and purity by flow cytometry, using FITC-conjugated anti-human α-smooth muscle actin (α-SMA) (Abcam, Cambridge, UK).

At the time of the experiments, no primary SCC cell line had been successfully established in our research group. Only human PAC cells and CAFs were available.

4.1.1 Experimental conditions

For each cell line, three parallels were seeded into three separate series (n=9). The cells were seeded onto six different plates prior to each experiment, three plates for normoxic conditions (6h, 12h, 24h) and three plates for hypoxic conditions (6h, 12h, 24h). Preceding each experiment, all plates were kept at normoxic condition (5% CO_2 and 21% O_2) for 48h to allow cell adherence and equilibrium. Subsequently, fresh starvation medium (0.5% FBS) was added to the wells and the cells were then exposed to either normoxia (21% O_2) or hypoxia (2.5% O_2). Hypoxia was maintained by continuously infusing nitrogen gas in the incubator (Hera Cell150, Thermo Scientific). The supernatant from each well was harvested at the end of each incubation period and subsequently centrifuged, transferred to new eppendorf tubes and stored in -80° C prior to analysis. We took two replicates of each sample to increase the precision of our estimates.

4.1.2 ELISA & DC Protein assay

ELISA (enzyme-linked immunosorbent assay) is a tool that uses antibodies and color change to quantify a specific protein in the sample. The VEGF-A and VEGF-C concentrations in the cell supernatants were quantified by R&D Systems Quantikine ELISA kit (Cat.no DVE00 and DVEC00, Quantikine ELISA kit, R&D Systems, Abingdon, UK). The principle of the ELISA Quantikine® assay can be divided into three steps. First, samples or standards were added to a microplate pre-coated with a capture antibody. Any VEGF-A/VEGF-C present in the sample/standard was bound by the immobilized antibody. Subsequently, unbound materials were washed away. Second, a horseradish peroxidase (HRP)-conjugated detection antibody was added, and bound to the captured VEGF-A/VEGF-C. Unbound detection antibody was subsequently washed away. Third, to quantify captured VEGF-A/VEGF-C, tetramethylbenzidine substrate solution was added to the wells, and a blue color developed proportionally to the amount of VEGF-A/VEGF-C present. Next, color development was stopped and the absorbance of the color was measured at 450 nm.

The VEGF-A and VEGF-C concentrations were normalized to total protein concentrations using the DC Protein Assay (cat.no 500-0116, Bio Rad, Hercules, CA, USA). The principle of the assay is based on two steps, where the proteins in the samples react with two reagents, which subsequently lead to color development. The absorbance of the color was measured at 750 nm.

4.2 NSCLC tissue samples

4.2.1 NSCLC patient cohort

Primary tumor tissue samples from 371 patients diagnosed with stage I-IIIA NSCLC (1990 to 2004) were collected retrospectively at the University Hospital of Northern Norway and Nordland Central Hospital. From the original cohort of 371 patients, 36 patients were excluded due to inadequate paraffin-embedded fixed tissue blocks (n=13), other malignancy within 5 years prior to diagnosis (n=13) or chemotherapy or radiotherapy prior to surgery. Consequently, 335 patients with complete demographic and clinicopathological data were included in the study (Figure 4).

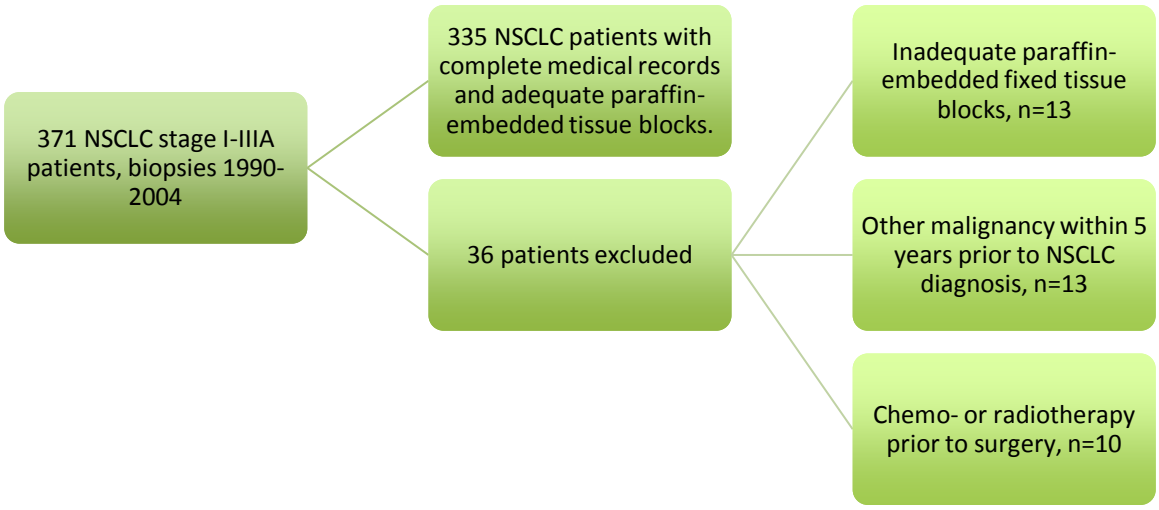

Figure 4. The cohort with criteria for inclusion and exclusion of the 371 stage I-IIIA NSCLC patients enrolled in the study.

4.2.2 Tissue microarray (TMA)

TMA is a procedure used to investigate the molecular profile of large tissue cohorts, in an efficient and cost-effective way. In one single operation, cores from hundreds of specimens, collected on a single slide, can be evaluated for biomarker expression simultaneously. It is possible to detect DNA, RNA and protein expression, using methods like immunohistochemistry or *in situ* hybridization. TMA have revolutionized the study on biomarkers.

Assembly of TMA blocks. Experienced pathologists selected the most representative areas of cancer cells and tumor stromal cells to be samples from each donor block. The sampling was done with a 0.6 mm needle by Manual Tissue Arrayer 1 (Beecher Instruments, Inc. WI, USA). The instrument samples cylindrical segments from donor blocks and places the cores into the predrilled recipient block. Two separate areas of cancer cells and tumor stromal cells, respectively, were sampled from each tumor. A total of 335x4=1340 cores were sampled and inserted into eight recipient blocks. In addition, 20 control specimens were collected in the same way from normal lung tissue and inserted in an additional control TMA-block. For the immunohistochemistry (IHC) and *in situ* hybridization (ISH) analyses, 4 µm sections were cut with the Rotary Microtome HM 355S (Microm International GmbH, Walldorf, Germany) and stained by specific antibodies or probes for molecular profiling of the NSCLC cohort (Figure 5).

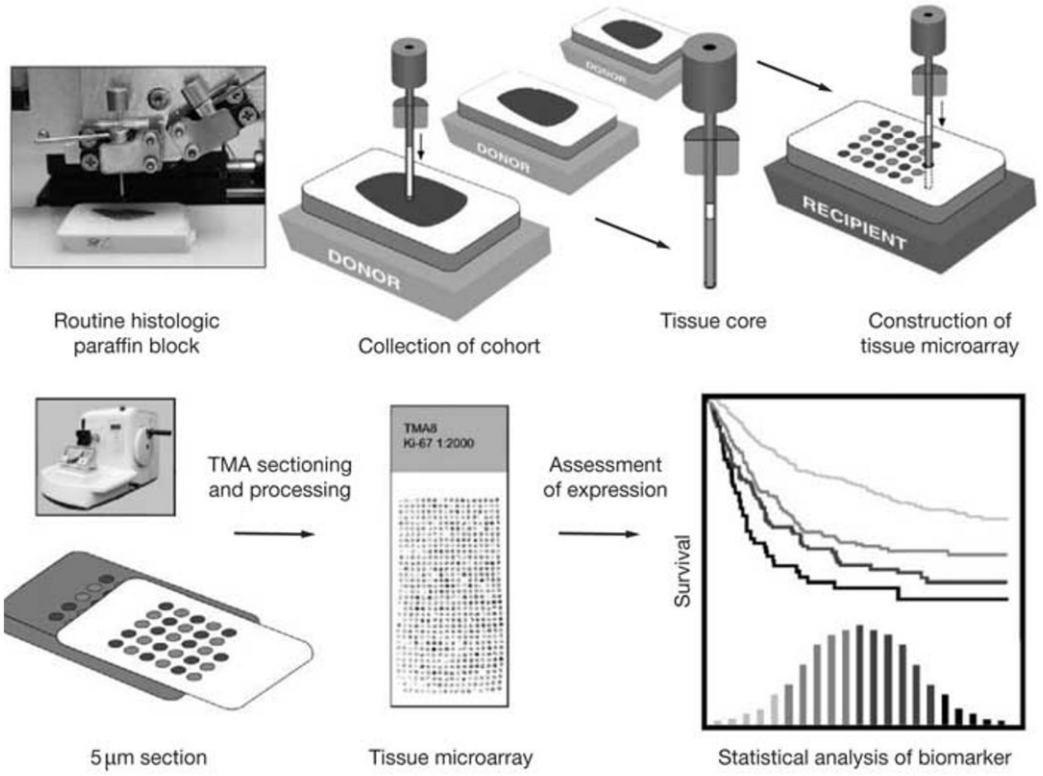

Figure 5. Schematic presentation of the construction of TMAs (adapted from [79]).

4.2.3 Immunohistochemistry (IHC)

IHC is a technique used to detect antigens in tissues with the use of specific antibodies that can be visualized through staining [80]. It is possible to detect antigens, like amino acids and proteins. IHC is an important tool for biomarker detection, histopathological diagnostics and research.

4.2.3.1 IHC procedures

In Table 4, an overview of antibodies used in the thesis is presented. Both manual and automated staining procedures were used in this thesis.

Principle of the manual staining procedure (VEGF-A, VEGF-C, LDH5, MCT2 and MCT3): The antigen recovery for the IHC procedure started with the removal of paraffin from the TMA sections by heating up the samples. Then, the slides went through multiple xylene washes and were subsequently rehydrated through graded washes of ethanol in water, ending in a final rinse in pure water. To remove the methylene bridges between the proteins, formed by the formaldehyde, the heat-induced epitope retrieval (HIER) was used; The TMA slides was placed in 0.01M citrate buffer (pH 6.0) and exposed to microwave heating at 450W.

For VEGF-A and VEGF-C the EnVision+ System-HRP (DAB) system was used (Cat.no K4011, Dako North America Inc., CA, USA). The principle of the assays was as follows; The TMA slides were incubated with the diluted rabbit primary antibody (antibody diluent: S080981, Dako North America Inc., CA, USA) followed by incubation with secondary antibodies that were conjugated with HRP-labelled polymer. Staining was completed by incubation with 3-3'diaminobenzidine (DAB) substrate-chromogen, which resulted in a brown-coloured precipitate at the antigen site.

For LDH5, MCT2 and MCT3 the VECTASTAIN® Elite ABC kit was used (Vector Laboratories, Inc., CA, USA). Non-specific staining was avoided by; 1) the TMA slides were incubated with blocking solution to quench endogenous peroxidase activity. 2) The TMA slides were incubated in diluted normal blocking serum that was prepared from the specimen the secondary antibody was made. The TMA slides were subsequently incubated with the diluted primary antibody, followed by diluted biotinylated secondary antibody solution. The slides were incubated in VECTASTAIN

Elite ABC Reagent followed by incubation in peroxidase substrate solution until desired stain intensity developed.

Principles of the automated staining procedure (HIF-1α, HIF-2α, GLUT1, CAIX, MCT1 and MCT4): The TMA slides were loaded onto the Ventana Benchmark XT (Ventana Medical Systems, Illkirch, France), and followed the ultraview DAB® procedure. The deparaffinization and antigen retrieval (HIER using standard Cell Conditioning Solution (CC1)) steps were user-defined, but were performed by the automated Benchmark system. Following this, the primary antibodies were applied, using a secondary antibody whereby the HRP-enzyme is directly conjugated. The primary-secondary antibody complex is visualized with hydrogen peroxidase substrate and DAB chromogen, resulting in a brown stain where the antigen is located in the cell. Finally, all slides were counterstained with haematoxylin to visualize the nuclei.

Table 4. Antibodies used in the IHC studies in the thesis.

Antigen	**Type**	**Manufacturer**	**Catalog #**	**Dilution**
HIF-1α	Mouse monoclonal	Novus Biologicals	NB100-131	1:3500
HIF-2α	Rabbit polyclonal	Abcam	ab199	1:40
GLUT1	Mouse monoclonal	Abcam	ab40084	1:500
LDH5	Rabbit polyclonal	Abcam	ab53010	1:100
CAIX	Rabbit polyclonal	Abcam	ab15086	1:500
VEGF-A	Rabbit polyclonal	Neomarkers	RB-1678	1:10
VEGF-C	Rabbit polyclonal	Zymed laboratories	18-2255	1:25
MCT1	Rabbit polyclonal	Millipore	AB3538P	1:75
MCT2	Goat polyclonal	Abcam	ab129290	1:150
MCT3	Rabbit polyclonal	Abcam	ab60333	1:50
MCT4	Rabbit polyclonal	Santa Cruz	sc-50329	1:200

IHC controls. Paper I; all applied antibodies had been subjected to in-house validation by the manufacturer for IHC analyses on formalin-fixed paraffin-embedded material. Paper II; the VEGF-A and VEGF-C antibodies were subjected to in-house validation by the manufacturer for IHC analysis on paraffin-embedded material. As negative controls, the primary antibody was replaced by with the primary antibody diluent. Paper III; all applied antibodies had been subjected to in-house validation by the manufacturer for IHC analysis on paraffin-embedded material. For MCT1 and MCT4, we additionally employed Western blots to verify the specificity of the selected antibodies. The size corresponded well with the predicted sizes, consistent with the data provided by the manufacturers. The observed relative expression levels of MCT1 and MCT4 proteins in the tested cell lines matched findings reported by others.

4.2.4 *In situ* hybridization (ISH)

We used ISH to determine the expression of miR-210, which visualizes the location of the miRNA in both its histological and cellular localization. This is in contrast to methods as Northern blotting and RT-PCR (real-time polymerase chain reaction) where cell homogenates are used. The ability to localize the miRNA was crucial since we were interested in investigating the impact of biological markers in both the cancer cell and tumor stroma compartment.

4.2.4.1 In situ hybridization procedure

The ISH procedure was done according to the "One-day microRNA ISH protocol". To obtain sensitive and specific detection of miR-210, some optimization of the protocol were done. The principal steps of the ISH procedure was as follows (for details, see the material and methods section in paper IV); First, the cores of the TMA was attached to Superfrost™ Plus Slides (Cat.no 12-550-15, Thermo Fisher Scientific, MA, USA) which, due to opposite electrical charge between the slides and the tissue, keeps the tissue adherent to the slides during the ISH procedure. With ordinary glass slides, about 50% of the tissue cores will partly or completely fall off[81]. In the next step the TMA slides was placed in xylene to remove the protective paraffin wax. Then, to remove the xylene, the slides were rehydrated in graded washes of ethanol (96-70%). Subsequently, the slides were washed in PBS before the tissue was digested by Proteinase K. Proteinase K is strong and well suited for tissue that has been fixed for a long time in formalin, which is true in our case. But one has to be careful that overdigestion does not occur, which will result in loss of tissue morphology. The hybridization step started after the slides was washed in PBS, dehydrated in graded washes of ethanol (70-96%) and air dried. The Locked Nucleic Acid (LNA) probes were denaturized by heating to 90°C. The TMA Superfrost™ Plus Slides were first covered with the probe and then with a polypropylene sterile coverslip and the hybridization reaction was allowed to proceed in a ThermoBrite hybridizer at 55°C. Then, the cover slip was removed and the slides went through stringent washes in pre-heated buffers. Next, unspecific binding was prevented by a blocking solution. The probe-miR-210 complex was visualized by alkaline phosphatase

(AP)-conjugated anti-DIG. Nuclear fast red was used as counterstain. During the whole procedure, a stringent approach was used to enable an RNase free work environment and RNase free solutions.

ISH controls. As negative control, we used the scramble probe obtained from Exiqon (Vedbeak, Denmark). The scramble probe have identical sequence as the miRNA cDNA, but the nucleotides are ”scrambled” at random so that the homology with the target sequence is very low [81]. As positive control, we used a small nuclear control probe; U6, hsa/mmu/rno (Exiqon). Weak or negative positive control may be due to degradation of tissue during the formalin fixation process or methodological errors. In addition, we used tissue known to be strongly negative and positive for miR-210. As negative tissue control we use samples from human epithelial ovarian cancer [82]. In the epithelial ovarian cancer cells, miR-210 was downregulated compared to adjacent normal epithelial cells (results not shown). As positive tissue controls, we used samples from breast cancer, pancreatic cancer, glioblastoma and clear cell carcinoma from kidney [78]. Further, two experienced pathologist performed a quality assessment of the ISH slides to secure specificity and avoid background staining.

4.2.5 Evaluation of staining

Apart from paper III, staining by IHC and ISH was evaluated by two experienced pathologists. Compared to digital image analysis, scoring by pathologists allows for distinction of biomarker expression in cancer cells versus tumor-associated stromal cells [83]. Further, it allows for future implementation into clinical practice, if the assays can be

validated. IHC scoring is a semiquantitative analysis, and to reduce subjectivity the slides were scored by two researchers (normally two pathologists; for paper III one pathologist and one trained physician). Interobserver reliability was calculated and found satisfactory.

4.2.6 Statistical analysis

All statistical analyses were done using the statistical package SPSS (Chicago, IL, USA); versions 15 (paper I), 16 (paper II) and 20 (paper III and IV).

Paper II. The ELISA data were expressed as the mean ± SEM. The numerical outcome from both groups (normoxia and hypoxia group) was tested statistically with paired-sample t-test. Significance was defined as $P<0.05$.

Paper I, III and IV. The chi-square test and Fishers exact tests were used to examine the associations between molecular marker expression and the clinicopathological variables. *r*-values are the Spearman's rank correlation coefficient. Univariate survival curves were drawn using the Kaplan-Meier method. Statistical significance between the high and low marker expression was assessed by the log-rank test. Disease-specific survival (DSS) was the endpoint in paper I, III and IV. DSS was defined from the date of surgery to the time of lung cancer death. The last DSS update was in January 2011. Variables with significant *P*-values ($P<0.05$) from the univariate analyses were entered into the multivariate Cox regression analysis (backward stepwise, probability for stepwise entry and removal was set at 0.05 and 0.10). In paper III and IV, two models were used. In Model 1, marker expressions in cancer and stromal cells were

tested simultaneously against the significant clinicopathological variables, while in Model 2 the co-expression variables were tested separately against the significant clinicopathological variables. A *P*-value <0.05 was defined as statistically significant. In paper IV, for a few of the tumor tissues, we only had one core for evaluation. Due to stringent assessment standards, we characterized these as missing as we did not want to extrapolate results from only one core.

5. MAIN RESULTS

5.1 Paper I

5.1.1 Correlations

A strong correlation was observed between HIF-2α and LDH5 in stromal cells (r=0.41, *P*<0.001) and GLUT1 in cancer cells and SCC histology (r=0.37, *P*<0.001).

5.1.2 Univariate analyses

For all NSCLC patients, ↑HIF-1α (*P*<0.001) and ↑GLUT1 (*P*<0.013) in cancer cells and ↓HIF-1α (*P*=0.028), ↓HIF-2α (*P*=0.001) and ↓LDH5 (*P*=0.011) in stromal cells correlated with poor DSS. For the SCC subgroup; ↑HIF-1α (*P*=0.001) in cancer cells and ↓HIF-1α (*P*=0.009) and ↓HIF-2α (*P*=0.005) in stromal cells correlated with poor DSS. In the AC subgroup, ↑GLUT1 (*P*=0.01) expression in cancer cells and ↓LDH5 in stromal cells (*P*=0.03) correlated with a poor DSS.

5.1.3 Multivariate analyses

For all NSCLC patients, ↑HIF-1α (HR: 2.3, 95% CI: 1.3-4.1, *P*=0.003) and ↑GLUT1 (HR: 2.0, 95% CI: 1.1-3.4, *P*=0.02) in cancer cells and ↓HIF-1α (HR: 1.8, 95% CI: 1.3-2.8, *P*=0.003) and ↓HIF-2α (HR: 1.8, 95% CI 1.2-2.8, *P*=0.006) in stromal cells were associated with poor DSS. In the SCC subgroup, ↑HIF-1α (HR: 3.3, 95% CI: 1.7-6.6, *P*=0.001) in cancer cells and ↓HIF-1α (HR: 2.1, 95% CI: 1.2-3.7, *P*=0.008) and ↓HIF-2α (HR: 2.3, 95% CI: 1.3-4.1, *P*=0.005) in stromal cells were associated with a poor DSS. In the AC subgroup, ↑GLUT1 (HR: 1.9, 95% CI: 1.0-3.6, *P*=0.046) in cancer cells and ↓LDH5 (HR: 2.3, 95% CI: 1.1-4.8, *P*=0.03) in stromal cells were associated with a poor DSS.

5.2 Paper II

5.2.1 VEGF-A response to hypoxia in AC and SCC cell lines

The major trend in the VEGF-A secretion after exposure to hypoxia over time, was a significant increase in both AC cell lines (H522 and PAC). In contrast, the SCC cell line (H520) had over time a reduction in VEGF-A secretion.

5.2.2 Comparison of the VEGF-A response to hypoxia between AC and SCC cell lines

After exposure to normoxia, the VEGF-A secretion from the SCC cell line (H520) was higher when compared to that of the AC cell line (H522). During hypoxia, the VEGF-A secretion was lower in the AC cell line (H522) than in the SCC cell line (H520) at 6 h (P<0.0001) and 12h (P=0.02), but not at 24h (P=0.75).

5.2.3 Comparison of the VEGF-A expression in tissues from patients with AC and SCC

In line with the cell line results, the overall expression of VEGF-A in the SCC tumors was higher when compared to AC tissues, but the results did not reach statistically significance (P=0.059).

5.3 Paper III

5.3.1 Correlations

A correlation between MCT1 in cancer cells and GLUT1 (r=0.38, *P*<0.001) and MCT1 in cancer cells and histology was observed, with high expression in 58% of SCC compared to 34% of AC patients (r=0.484, *P*<0.001).

5.3.2 Univariate analyses

↑MCT1 expression in cancer cells (*P*=0.021) and ↑MCT2 (*P*=0.006) and ↑MCT3 (*P*=0.020) expression in stromal cells and the co-expression marker ↑MCT1 in cancer cells + ↑MCT4 in stromal cells (*P*=0.006) correlated with a favorable outcome.

↑MCT1 in stromal cells (*P*=0.003) and ↑MCT4 in cancer cells (*P*=0.027) and the co-expression variables ↑GLUT1 in cancer cells + ↑MCT1 in stromal cells (*P*=0.001), ↑GLUT1 + ↑MCT4 in cancer cells (*P*=0.003) and ↑MCT4 in cancer cells + ↑MCT1 in stromal cells (*P*=0.009) was associated with a poor DSS.

5.3.3 Multivariate analyses

↓MCT1 in cancer cells (HR: 1.9, 95% CI: 1.3-2.8, P=0.001), ↓MCT2 in stromal cells (HR:2.4, 95% CI:1.5-3.9, *P*<0.001) and ↓MCT3 (HR: 1.9, 95% CI: 1.1-3.5, *P*=0.031), ↑MCT1 in stromal cells (HR:1.7, 95% CI: 1.1-2.7, *P*=0.016) and the co-expression variables ↑GLUT1 in cancer cells + ↑MCT1 in stromal cells (HR: 7.3, *P*=0.016) and ↑GLUT1 + ↑MCT4 in cancer cells (HR: 3.3, *P*=0.031) were all significant and independent prognostic factors for poor DSS.

5.4 Paper IV

In a pilot screening of the expression of 281 miRNAs by our group, tumor tissues from 20 NSCLC patients, 10 worst and 10 best prognosis, as well as tissue from 10 normal lungs were used [84]. The hypoxia related miR-210, was found to be upregulated in tumor tissue compared to normal tissue. As a consequence, we sought to study the prognostic impact of miR-210 in our large NSCLC cohort. miR-210 expression was evaluated in both cancer and stromal cells.

5.4.1 Correlations

We only found modest to weak, although highly significant, correlation between miR-210 in stromal cells and the hypoxic and angiogenic markers HIF-1α (r=0.161, *P*= 0.006), HIF-2α (r=0.185, *P*= 0.002) and PDGFRα (r=0.210, *P*<0.001) in stromal cells.

5.4.2 Univariate analyses

High expression of miR-210 in cancer cells (*P*=0.039) was significantly associated with a favorable outcome. High expression of miR-210 in stromal cells (*P*=0.008) were also significantly associated with a positive outcome.

5.4.3 Multivariate analyses

Low expression of miR-210 in stromal cells (HR: 1.9, CI 95%: 1.1-3.0, *P*=0.013) was significantly and independently associated with a worse prognosis. miR-210 expression in cancer cells versus prognosis did not reach statistical significance.

6. DISCUSSION

6.1 Methodological considerations

6.1.1 Study designs

A great strength of this thesis is the use of different study designs (experimental and observational cohort) to investigate the hypoxia response pathways in NSCLC. Besides, a broad range of methods have been used (cell cultures, ELISA, TMA, IHC, ISH and Western Blot) enabling us to answer the aims stated.

6.1.2 Experimental study: Cell lines

Experimental studies comprise studies where the investigator intervenes in some way to affect the outcome. The strength of these is the control of factors which may otherwise bias the outcome, providing more robust evidence when testing the hypothesis.

Continuous cell lines are commonly used as *in vitro* models in cancer research[85]. Their advantages are several; they are easy to handle, and represent an unlimited self-replicating source. Further, they are relatively homogenous and are easily stored in frozen stocks. The disadvantage of continuous cell lines are that they are susceptible to genotypic and phenotypic changes as passages increases, making them less suitable as a model for the tumor type they originated from.

Primary cell lines, on the other hand, may be an alternative source as an *in vitro* model of cancer. These cell cultures derive directly from in-house freshly resected tumors, and hence the biological response observed in an experimental setting may be

closer to an *in vivo* situation than the one obtained with a continuous cell lines. Further, detailed information about its histopathology is easily retained. The primary cell lines can be established as an explant culture (from small tissue parts, mixed cell populations grow out) or culture of individual cells, which is more desirable as you separate the epithelial cells from stromal fibroblasts, and thereby avoid that they are outgrown by fibroblast, which more easily adapt to *in vitro* conditions. Disadvantages of primary cell lines, which are also a disadvantage of continuous cell lines, is that they may behave differently *in vitro* as compared to their response in the tumor. This is due to cell-cell interactions in the tumor, which is lost in this *in vitro* model. Further, primary cell lines of epithelial cancer cells have slow doubling time and a short lifespan *in vitro*. This makes use of them problematic, especially for experiments running over a long time or where you need a large number of cells.

6.1.3 Observational study: Cohort study

An observational study is one where the investigator does not influence the outcome, but only observes what happens. Examples of observational studies are case-control and cohort studies. Usually, a cohort study includes a group of individuals that are usually followed over time (prospective cohort) or observed retrospectively as in this thesis (historical cohort). The demographic, clinical and histopathological data was obtained from medical records at the university hospital, local hospital and/or the patient's general practitioner. The benefits of a retrospective/historical cohort are lower costs and more quickly available data when compared to prospective studies. The

disadvantage may be lower data quality and certainly the impossibility to do randomized studies. In addition, retrospective single center trials may be especially associated with bias due to selection, the nature of the center and inadequate records.

6.1.4 TMA

Like all methods, TMA have its strength and weaknesses (Table 3). The TMA technology has several advantages, compared to using whole sections of tissues with one slide for every patient. First, TMA is a time saving procedure for the technician and scorer(s). As an example, our material was composed of eight slides, instead of 335, and IHC and ISH can be performed on these eight slides in one single operation. Since TMA cores are from the most representative areas of each donor block (secured by a highly experienced pathologist). The experienced scorer will immediately see the areas of cancer and tumor stromal cells in the microscope, and do not have to locate suitable areas for scoring on a whole section slide. Second, TMA reduces costs significantly because of the significantly lower amount of antibodies/probes and other reagents and material needed when processing and cutting eight blocks instead of 335. Third, TMA slides are well suited for exchange between research groups. It is easy to compare scoring of slides and choice of cut-off values. Unstained slides make it possible to compare IHC and ISH procedures.

On the other hand, producing and cutting TMA slides is technically challenging and is therefore dependent on a committed and well-trained technician. Further, by collecting tissue samples dating over a large time span, the chances of different

techniques in tissue processing (e.g. fixation) increases. Thus, it is important that the tissues included have been processed in a similar and reproducible way during the actual time period.

Representativity is a common concern when comparing TMA with whole tissue slides, but studies addressing this issue have shown good correlation between scoring of whole sections and TMA slides when it comes to evaluating biomarker expression in larger cohorts[86]. In agreement, representativity of biomarker expression studied in TMA compared to regular sections is reported to be >90-95%[87]. In addition, representativity can be improved by increasing the number of cores from each patient[88]. Still, TMAs are not suitable for diagnostic purposes, as the cores will not reveal possible variations in heterogenous tissues[89]. Results from TMA studies should be prospectively validated in actual patient settings before implementing them in a clinical setting.

Table 3: Strength and weaknesses of TMA

Strengths	Weaknesses
Saves time	Possible technically challenging
Reduces costs	Lower accuracy when heterogenous expression of biomarkers
Saves tissue	Variation through the core
Enables study of larger cohorts	Not suited for individual diagnosis
Standardized staining conditions	
Research collaboration through sharing slides	

6.1.5 IHC

Polyclonal and monoclonal antibodies are two different antibody types with both strengths and weaknesses. Monoclonal antibodies are homogenous immunoglobulins directed against a single epitope of the antigen. The monoclonal immunoglobulins are produced by a single B-cell clone and are therefore identical. To mass-produce the monoclonal antibodies, the B-lymphocytes are isolated and fused with immortal myeloma cell lines and subsequently injected into the peritoneal cavity of an animal or placed in a bioreactor system. Monoclonal antibodies have the advantage of lot-to lot consistency, since its production depends on an immortal cell line and not on the life of the animal as with the polyclonal antibody production.

Polyclonal antibodies are directed against various epitopes and are therefore a heterogenous mixture of antibodies. The polyclonal antibodies are produced by immunizing the animal with the antigen that the antibody produced is going to detect. The blood from the animal is collected three to eight months later and the antibody is purified. The polyclonal antibodies produced have slightly different affinities and specificities against the antigen. The polyclonal antibodies are more robust, and less false negative results are produced since the antibodies recognize various epitopes on the antigen. On the other hand, the same feature increases the chance of cross reactivity.

6.1.6 ISH

In general, miRNAs are challenging to study. This is partly due to their short length (19-23 bp), which leads to a melting temperature of the miRNA/cDNA probe hybridized complex which may be too low for their detection[81]. LNA is a nucleic acid analog, in which the ribose ring is locked in an optimal confirmation for a higher base paring affinity to the miRNAs compared to DNA and RNA[90]. Further, to enable specific identification, the design of LNA oligos allow for optimization of increased mismatch discrimination. LNA probes have shown great advantages in numerous platforms, including ISH.

6.1.7 Determination of cut-off values

The expression of the biomarkers in this thesis varied over a continuous scale, and the choice of cut-off values is an important and difficult issue. To standardize cut-off values for each biomarker is challenging, due to variation in methods used among research groups, including differences in tissue handling, antibody type and manufacturer and assessment of biomarker expression. Many research groups have used mean value as the cut-off point, which makes the results easier to reproduce, but the chance of false negative results increase (type 2 errors). Since the risk of missing biologically important mechanisms increases by using mean values, we have used the cut-offs for each biomarker that gave the most significant difference in DSS between the subgroups, while maintaining large enough subgroups. By using this approach we aim to identify the biologically significant cut-offs, but the risk of getting false positive results increases

(type 1 errors). Therefore, it would be of great value to get an independent validation of our results, and hence we have initiated collaboration with other lung cancer research groups to establish validation sets. In addition, we are expanding our own material so that tissue specimens from one of the institutions can be used as a validation set for the training set.

6.2 DISCUSSION OF MAIN RESULTS

Paper I

Hypoxia in cancers is by itself associated with a poor outcome[52]. HIF-1α, the master transcriptional regulator of the hypoxic response, is reported to be an independent prognosticator for poor survival in several cancers[91].

To our knowledge, we are the first to report that high expression of HIF-1α in NSCLC cancer cells is an independent prognostic marker for poor survival. Giatromanilaki et al. only found a trend towards an association with poor survival and high HIF-1α expression[92]. A similar negative prognostic impact by high HIF-1α expression in NSCLC tumors was found in univariate analysis by Kim et al.[93]. An explanation of HIF-1α's negative prognostic impact in NSCLC cancer cells may be that it serves as surrogate biomarker of hypoxia, which is associated with a poor outcome[52]. However, the fact that a stringent association between HIF-1α and hypoxia under standardized *in vitro* conditions is well established does not prove that this association is directly transferable to the clinic[94].

In contrast to the expression in NSCLC cancer cells, high HIF-1α and HIF-2α expression in tumor-associated stromal cells correlated with a favorable prognosis. The HIF-1α finding in tumor stroma is in agreement with a previous study in breast cancer showing that high HIF-1α expression in the stroma compartment was associated with a better prognosis[95]. Our data may be a result of *in situ* immunity, since we found a weak to moderate correlation between lymphocytes and hypoxia markers. In contrast to this

hypothesis, Lukashev et al. have reported that hypoxia, and subsequently HIF-1α activity in T lymphocytes, can protect cancer cells from damage by immune cells[96].

In the subgroup analysis according to histological entities, HIF-1α in cancer and stromal cells and HIF-2α in stromal cells showed a prognostic role in SCC, but not in AC. SCC tumors are known to exhibit more necrosis, probably due to hypoxia, than other NSCLC subtypes. In support of this theory, a metaanalysis by Ren et al. showed that HIF-1α expression was significantly higher in SCC than in AC[97].

Our data shows that high GLUT1 expression in NSCLC cancer cells has an independent negative prognostic impact. Consistently, Younes et al. and Minami et al. have previously reported associations between high GLUT1 expression in NSCLC cancer cells and poor survival[98,99]. However, Nguyen et al. did not find such an association[100]. High cancer cell GLUT1 expression had a significantly stronger prognostic impact in the AC than in the SCC subgroup, even though there was a higher proportion of SCC tumors' expressing the marker. Consistent with our observation, Meijer et al. found a higher proportion of SCCs than ACs expressing GLUT1, and that a prognostic impact by GLUT1 expression only was seen in ACs[101]. Besides, the study by Minami et al. confirms that GLUT1 is an independent prognostic factor in ACs of the lung[99].

Our data show that high LDH5 expression in stromal cells indicated a favorable prognosis. This finding may be explained by the observation that the stromal cells of these patients did not show a complementary metabolic profile between cancer cells and tumor-associated stroma as hypothesized by Koukourakis et al.[72].

Our study provides additional evidence for why increased focus on histological subgroups in NSCLC and more personalized therapy is important along with the introduction of new molecular targeted drugs[13]. Further, our observations highlight the complexity of cancer biology, emphasizing that drugs with molecular targets may give diverging effects in cancer cells versus the cancer-related stromal compartment.

Paper II

To our knowledge, the *in vitro* VEGF-A response to hypoxia in AC compared to SCC has not been investigated previously. Our main finding is that the AC subgroup responded to hypoxia with increased VEGF-A secretion, while the SCC cell line reduced its VEGF-A secretion under hypoxic conditions.

The large number of parallels in our *in vitro* experiment is a major strength of our data. In addition to the commercial cell lines, we included one in-house PAC cell line and one in-house CAF cell line. Our stringent approach to simulate tumor hypoxia levels (≤2% O_2) in our *in vitro* environment makes the experimental set up highly relevant. Another strength of the *in vitro* results is that our PAC cell line showed similar responses to hypoxia as the commercial continuous AC cell line. We had obtained both ethical approvement and patient consent to establish primary cell cultures. An obstacle was, at the time, limited tissue availability, making only one PAC culture and primary CAF culture available at time of this study. Only one SCC cell line (H520) was available at the time of the experiments.

To our knowledge, we are the first to report the VEGF-A response of a SCC cell line to hypoxia. Fukuyama et al. have previously investigating the cytokine production (including VEGF-A secretion) in NSCLC SCC and AC cell lines, but this was performed under normoxic conditions only[102]. We have confirmed that AC cell lines respond to hypoxia with an increase in VEGF-A secretion, in agreement with three previously published studies[103-105]. When comparing the VEGF-A response between the cell lines, we observed an overall higher VEGF-A expression in SCC compared to AC under hypoxic conditions. But surprisingly, the SCC cell line response to hypoxia was a down-regulation of VEGF-A. To further investigate the observed difference in *in vitro* VEGF-A expression in AC versus SCC, we used a large number of NSCLC tumor tissues to assess whether there is an overall difference in the VEGF-A expression between these histologic subtypes. We found that the VEGF-A expression tended to be higher in SCC than in AC tissues ($p=0.059$), which is consistent with our *in vitro* result. Our results are hypothesis-generating and points to a potential difference between SCC and AC with respect to an angiogenic response to hypoxia. This finding may explain the observation that SCCs appear to be more hypoxic and necrotic than ACs[106]. We hypothesize that an overall higher VEGF-A expression might disrupt the balance of pro-angiogenic and anti-angiogenic signals, resulting in distorted angiogenesis with abnormal vessel formation or no vessel formation at all. In support of this theory, studies on mean vascular density (MVD), which reflects in situ angiogenesis, have reported higher MVD in AC than in SCC of the lung[101,107-109]. Further, a study published in Radiology in 2012 showed that the flow-extraction product was significantly higher in AC than in SCC tumors, indicating that

AC has a more functional vasculature than SCC[110]. There is an apparent need for further studies to confirm our observations. Since SCC histology appears to be a subpopulation with inferior response to bevacizumab, when compared to AC, this difference may be related to difference in the VEGF-A secretion between these two major NSCLC cell types[13]. Further studies comparing angiogenic biomarkers in NSCLC histological subgroups are needed, as novel anti-angiogenic therapies are emerging.

Paper III

Our finding of an association between high MCT1 expression in NSCLC cancer cells and a favorable outcome was surprising. A few studies have reported that high MCT1 expression is associated with an unfavorable outcome when expressed alone or co-expressed with CD147 or p53[75,111,112]. Since 1) MCT1 is capable of transporting lactate both in and out of cells and 2) lactate imported by MCT1 is shown to induce a gene expression profile associated with a beneficial clinical outcome, we hypothesize that MCT1 in NSCLC import lactate. In other cancers where MCT1 is shown to have a negative prognostic impact, MCT1 may export lactate. Accordingly, the prognostic impact may diverge between different cancer types[71,113]. Besides, the positive prognostic impact by MCT1 expression in NSCLC cancer cells might be due to the accordingly decreased acidosis in the microenvironment. An acidic environment is associated with breakdown of the extracellular matrix, a process which favors invasiveness[114]. But if this is so, one would expect MCT1 expression also in stromal cells to be associated with a positive prognostic impact. Clearly, functional studies clarifying

MCT1s role in NSCLC is needed since MCT1 together with MCT4 has been observed to promote cancer cell invasion in lung cancer[115].

High MCT1 expression in stromal cells was associated with a poor outcome. This is supported by studies linking MCT1 expression with tumor angiogenesis activation[116,117]. In addition, MCT1 in stromal cells is linked to the contribution to a metabolic co-operation of lactate homeostasis between recruited stromal cells and glycolytic cancer cells[118]. This is also in agreement with our results.

In agreement with NSCLC data from Meijer et al., ↑GLUT1 + ↑MCT4 in cancer cells was associated with a poor outcome[101]. While Meijer et al. found this association only in AC; we observed the same significant impact in all histological subgroups, possibly due to our larger study cohort.

To our knowledge, we are the first to provide strong evidence supporting the metabolic co-operation theory of Koukourakis et al. as the co-expression of ↑GLUT1 in cancer cells + ↑MCT1 in stromal cells had a synergetic, strongly negative prognostic impact[72,119]. Interestingly, Fiaschi et al. observed an alternative metabolic co-operation between cancer cells and CAFs[62]. They found that cancer cells can allocate Warburg metabolism to CAFs, and subsequently take advantage of their lactate production to grow in an environment low on glucose, hence symbiotically adapting with stromal cells to low glucose availability.

May MCT1 be a potential therapeutic target in NSCLC in light of our results? The MCT1 inhibitor AZD3965 is in Phase I clinical trials in the UK[120]. According to Miranda-Concalves et al. this should be a promising treatment strategy as MCT1 inhibition will

indirectly starve latent malignant hypoxic cancer cells exhibiting both anti-tumor and anti-angiogenic activity[121].

According to our data, however, inhibition of MCT1 may be contraindicated as MCT1 in NSCLC cancer cells contribute to a tumor phenotype with a better prognosis. A optimal treatment approach might be to selectively target MCT1 in stromal cells. In fact, inhibition of MCT1 expressed in endothelial cells has recently been suggested[116].

Paper IV

The observed positive prognostic impact of high miR-210 expressed in NSCLC cancer cells are in agreement with studies in renal cell carcinomas and soft-tissue sarcoma[54,122]. In contrast, high expression of miR-210 in head and neck cancer and breast cancer has been associated with poor outcome[123,124]. A possible explanation for the inconsistent prognostic impact of miR-210 across different tumors may be miR-210 regulatory effects varying according to cancer type.

Rationales for a positive prognostic impact by miR-210 have emerged from several functional studies in cancer cells. miR-210 is observed to inhibit cell proliferation in ovarian cancer cell lines and esophageal SCC[82,125]. When human pancreatic or head and neck cancer cells ectopically expressing miR-210 were implanted in immunodeficient mice, miR-210 repressed initiation of tumor growth[126]. Consistently, miR-210 up-regulation in lung cancer cell lines inhibited proliferation and growth[127].

With respect to tumor stromal cells, possible explanations for the association between high miR-210 expression and positive outcome can be found in the following

two studies[128,129]. Torti et al. found that miR-210 is important for iron uptake in stromal cells, and that iron in these cells act as iron-pools which rapidly dividing cancer cells can feed on. Indirectly, high miR-210 can inhibit cancer cells proliferation due to iron depletion[128]. Faraonio and coworkers found that high expression of miR-210 in normal fibroblasts promote a senescent phenotype and reduce cell proliferation[129]. CAFs are known to exhibit pro-tumor activity by delivering paracrine oncogenic signaling to cancer cells and endothelial cells, and subsequently induce angiogenesis[130]. If high expression of miR-210 in CAFs can induce a senescent phenotype and reduce cell proliferation, is yet to be confirmed. If so, this would provide additional support for miR-210's positive prognostic impact in tumor stroma.

Since miR-210 is reported to be "the micromanager of the hypoxia pathway", we wanted to explore its associations with previously studied hypoxic and angiogenic markers in the same NSCLC cohort. We found only a few significant and only modest correlations. This might be explained by hypoxia being a dynamic process, making ISH and IHC "snapshots" rather unreliable for determining the functional correlation of these markers[131].

According to our data, miR-210 is a *prognostic* biomarker in NSCLC. In general, miRNAs may be promising therapeutic agents in the future. In order to inhibit tumor growth, a tumor suppressor miRNA may be reintroduced in cancer cells, possibly given as a systemic drug[132]. Introduction of miR-210 in NSCLC patients expressing low tumor levels may be a potential future treatment approach, especially since miR-210 is associated with a positive prognostic impact in both cancer and stromal cells.

But first, functional studies are warranted, especially in light of a recent publication stating that miR-210 may be a negative *predictive* marker for radiotherapy in both lung cancer and hepatoma cell lines[133]. We explored the prognostic impact in the radiotherapy subgroup of our cohort (data not shown). In this subgroup, however, miR-210 had a positive prognostic impact. In a clinical NSCLC study, Li and coworkers showed a significant correlation between increased serum levels of miR-210 and more advanced clinical stage and regional lymph node involvement[134]. In our cohort, however, we found only weak or insignificant correlations ($r<0.2$) between miR-210 expression and clinicopathological factors.

7. CONCLUSIONS AND IMPLICATIONS FOR FURTHER RESEARCH

We show that high HIF-1α and GLUT1 expression in cancer cells are independently and significantly associated with a poor prognosis in NSCLC. We report that HIF-1α and HIF-2α expression in stromal cells are independently and significantly associated with a favorable outcome in NSCLC. We are the first to show that the prognostic relevance of the hypoxia-related markers have different impact according to NSCLC histology.

In NSCLC, we are the first to report that *in vitro* VEGF-A secretion in response to hypoxia differ between AC and SCC cell lines. Even though the overall secretion of VEGF-A was higher in the SCC cell line (a trend confirmed in our TMA database), the response to hypoxia in the SCC cell line was a downregulation of VEGF-A secretion when compared to the normoxic condition. We hypothesize that AC might have a more balanced and functional angiogenesis, compared to SCC. Further functional studies to confirm our findings are needed.

We are the first to show that in NSCLC, MCT1 has a divergent independent and significant prognostic impact in cancer cells versus stromal cells. In cancer cells, high expression of MCT1 was associated with a favorable outcome, while high expression in stromal cells was associated with a poor outcome. Moreover, we found a substantial synergistic prognostic effect of the co-expressions of GLUT1 + MCT1 and GLUT1 + MCT4.

We found that miR-210 is associated with a positive prognostic impact in both NSCLC cancer and stromal cells. In stromal cells, high expression of miR-210 was associated with an independent and significantly favorable outcome in NSCLC.

In this thesis, I have sought to expand the knowledge on how hypoxia influences the tumor cell phenotype and hence the prognostic impact in NSCLC. I have dedicated my research to both the malignant cell and the resident tumor stromal cells, taking into account the budding current knowledge on how stromal cells affect cancer cells. Establishing a better understanding of the molecular details of this tumor-stroma interplay may lead to new treatment strategies.

Hopefully, our research on hypoxia in NSCLC may help providing a conceptual framework for the interpretation of the complex biology of NSCLC, and that further progress may lead to therapeutic advances in this field.

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