Hypoxic markers and prognosis in non-small cell lung cancer (NSCLC)

A translational research study utilizing immunohistochemistry on tissue microarrays for evaluation of endogenous markers associated with hypoxia and angiogenesis

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TABLE OF CONTENTS

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LIST OF PAPERS

Paper I **Andersen S, Eilertsen M, Donnem T, Al-Shibli K, Al-Saad S, Busund LT and Bremnes RM.** Diverging prognostic impacts of hypoxic markers according to NSCLC histology. Lung Cancer. 2011 Jun; 72(3):294-302.

Paper II **Andersen S, Donnem T, Al-Shibli K, Al-Saad S, Stenvold H, Busund LT , Bremnes RM.** Prognostic Impacts of Angiopoietins in NSCLC Tumor Cells and Stroma: VEGF-A Impact Is Strongly Associated with Ang-2. PLoS One. 2011; 6(5):e19773. Epub 2011 May 16

Paper III **Andersen S, Donnem T, Stenvold H, Al-Saad S, Al-Shibli K, Busund LT , Bremnes RM.** Over expression of the HIF hydroxylases PHD1, PHD2, PHD3 and FIH are individually and collectively unfavorable prognosticators for NSCLC survival. PLoS One. 2011;6(8):e23847. Epub 2011 Aug 22

LIST OF ABBREVIATIONS

ANG	= Angiopoietin
BCL2	= B-cell lymphoma 2
BNIP3	= BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
CAF	= Carcinoma associated fibroblasts
CAIX	= Carbonic anhydrase IX
CXCR4	= Chemokine receptor type 4
DLL4	= Delta-like ligand 4
DEC1	= Differentiated embryo-chondrocyte expressed gene 1
DSS	= Disease-specific survival
ECM	= Extracellular matrix
EGFL7	= Epidermal growth factor-like domain multiple 7
EGFR	= Epidermal growth factor receptor
EML4-ALK	= echinoderm microtubule-associated protein-like 4 (EML4) and the anaplastic lymphoma kinase (ALK)
EMT	= Endothelial-mesenchymal transition
EPO	= Erythropoietin
FGF	= Fibroblast growth factor
FIH	= Factor inhibiting HIF
GLUT1	= Glucose transporter 1
HIF	= Hypoxia induced factor
HR	= Hazard ratio
HRE	= Hypoxia responsive element
IFP	= Interstitial fluid pressure
IHC	= Immunohistochemistry
LDH5	= Lactate dehydrogenase 5
MMP	= Matrix metalloproteinase
NSCLC	= Non-small cell lung cancer
PDGF	= Platelet derived growth factor
PHD	= Prolyl hydroxylase
PLGF	= Placental growth factor
QOL	= Quality of life
SCLC	= Small cell lung cancer
TAD	= Transactivation domain
TAM	= Tumor-associated macrophage
TKI	= Tyrosine kinase inhibitor
TMA	= Tissue microarray
US	= United States
VEGF	= Vascular endothelial growth factor
ZEB	= Zinc finger E-box-binding homeobox 1

1 INTRODUCTION

The need for research in lung cancer is obvious when you look at cancer statistics. In Norway it is the 3rd most common cancer by incidence, but beyond competition killer number one. Survival has improved markedly for many of our more prevalent cancers, illustrated by a national increase in 5-year survival for all cancers from around 40% to above 65% during the last 30 years. Disappointingly, lung cancer patients have not enjoyed a significantly improved prognosis today compared to 3 decades ago and the survival is poor (below 15% 5-year survival).(1) From years of clinical work with different cancers, advanced lung cancer has therefore marked me due to the gravity of this disease.

Surgery, chemotherapy and radiotherapy have been the main pillars of cancer treatment. Some of the recent improvement in survival of other cancers can be attributed to novel treatment therapies. Such therapies increasingly target specific molecules involved in cancer progression and metastasis. Development and clinical introduction of targeted therapies takes several years. The important initial step is target definition. Experimental research continuously identifies new and potentially important molecules in cancer progression. The next step is to identify the significance of these molecules in different cancers. It must be determined to what extent the target is present in cancer. If present, a hypothesis of involvement in cancer progression can be claimed if the presence of the target is found to have a prognostic impact. Prognostication by molecular markers is also important as this may identify subgroups of patients in need for additional treatment or not, which traditional clinicopathological prognosticators have not been able to identify.

In 2004 a translational research strategy in non-small cell lung cancer (NSCLC) was established at the University of Tromsø/University Hospital North Norway by my mentors, Professors Roy M. Bremnes and Lill-Tove Busund. Since, a continuous effort has resulted in

several publications and dissertations by our group. One of the early identified fields of interest was hypoxia and angiogenesis, which is the theme of this dissertation.

The first paper in this dissertation report on the Hypoxia Induced Factors (HIFs) and some of the HIF controlled molecules in subgroups of histology. The HIFs were of great interest since they are at the heart of the adaptive mechanisms cancer cells must master for survival and proliferation.

The second paper was directed more towards angiogenesis and report on the prognostic significance of the angiopoietins (Angs) 1, 2, 4, their receptor Tie-2 and co-expression subgroups of Ang-2 and VEGF-A expression. We specifically wanted to look at subgroups as we have already published on VEGF-A in NSCLC and there is a close interplay between VEGF-A and angiopoietins in angiogenesis and there is targeted therapy against VEGF-A.

The third paper shifted the focus back to hypoxia and reports on the prognostic significance of oxygen sensors in cells, namely HIF hydroxylases. We were able to access validated antibodies from a collaborating research group in Oxford for these biomarkers which there is limited data on the prognostic impact of.

All of the investigated molecules have been proposed as potential targets in cancer and for some molecules there is ongoing research with directed therapies towards these targets. Altogether, this dissertation covers protein expression data regarding these molecules and their prognostic impact in NSCLC. These data will potentially improve prognostication of operable NSCLC patients and aid the more enduring efforts of establishing and implementing novel treatments for patients with this highly deadly disease.

2 BACKGROUND

2.1 Lung cancer

2.1.1 Epidemiology

Lung cancer is by far the number one killer among cancers in the western world, independent of gender, and in the US it accounts for more cancer related deaths than prostate, colorectal and breast cancer combined.(2;3) In Norway, there was diagnosed 1519 and 1129 new cases in men and women in 2009, almost 6 persons die every day of lung cancer and nearly 20% of all cancer related deaths were due to lung cancer.(1) In the US, lung cancer mortality has already dropped sharply for men and has reached its peaking threshold for women, whereas in Norway the peaking threshold for men has just been reached and there is still a solid increase for women (Figure1).

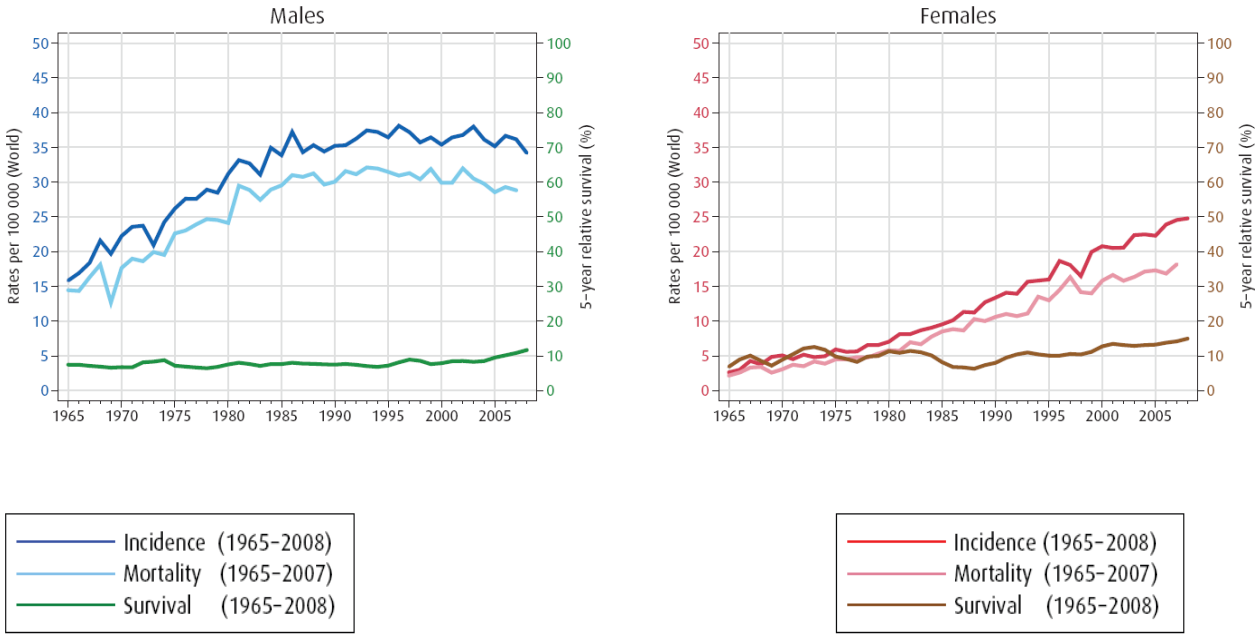


Figure 1: Trends in incidence and mortality rates and 5-year relative survival proportions (adapted from www.kreftregisteret.no; cancer in Norway 2008.)

As to the causes of lung cancer, cigarette smoking is by far the most important etiologic factor due to the high content of carcinogens in cigarette smoke and tobacco

products is generally by far the largest voluntarily source of human exposure to carcinogens.(4) Other known carcinogens for lung cancer are asbestos, radon, arsenic, cadmium and chromates.(5) Never smokers account for up to 25% of lung cancer patients and has recently been suggested as a separate entity due to differences in epidemiological, clinical and molecular characteristics.(6) The incidence patterns of lung cancer are highly linked to smoking habits in the population. Never smoking men have been estimated to have a risk of 0,2% to develop lung cancer, whereas current smokers was estimated to have a risk of 15,9% % to develop lung cancer by 75 years of age in the UK.(7) In Norway we have had a steady decline in daily smokers (Figure 2), but the decline started later than in the, probably explaining the different trend in incidence patterns between Norway and US. Regarding survival of lung cancer in Norway there seems to be a recent promising, but minor increase in the otherwise depressing 5-years survival (Figure 1).

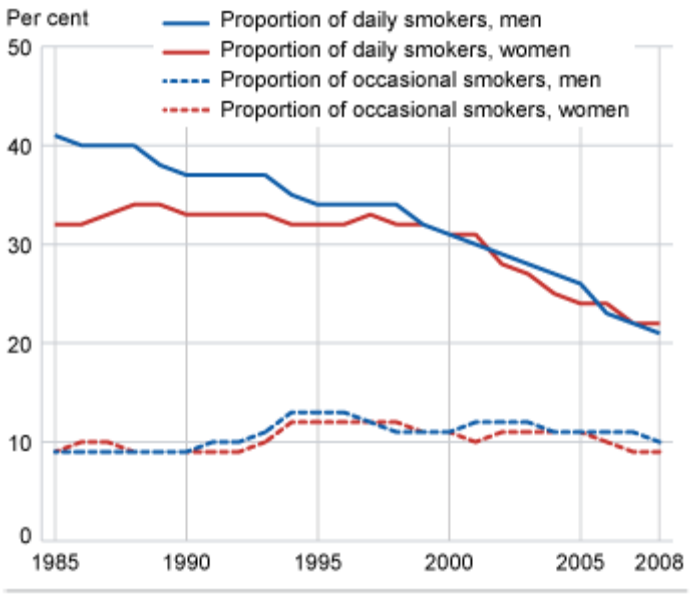


Figure 2: Female and male daily smokers and occasional smokers in Norway 16-74 year-olds. (adapted from www.kreftregisteret.no; cancer in Norway 2008.)

2.1.2 Histopathology

Morphologically and clinically, lung cancer is usually divided in two main entities: Non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is the largest group (80% of lung cancers) and the major morphologic subtypes are adenocarcinoma (AC), squamous cell carcinoma (SCC) and large cell carcinoma (LCC).(8) Internationally and nationally there has been a shift from squamous cell carcinomas to adenocarcinomas as the most frequent NSCLC subtype, probably due to increasing sales of filter cigarettes. Adenocarcinoma is also the commonest subtype among never-smokers.

Traditionally there has been little treatment-related interest in histological subgroups of NSCLC. However, this stand is challenged by the implementation of novel therapies where efficacy and side effects is highly dependent on histological subgroups.(9;10) Pemetrexed (Alimta®), which is a new multitargeted antifolate agent, has shown efficacy in adenocarcinoma and large-cell carcinoma, but not in squamous cell carcinoma.(11) The vascular endothelial growth factor (VEGF) antibody bevacizumab (Avastin®) have a modest efficacy in non-squamous NSCLC, but the drugs` efficacy was not tested in squamous cell histology due to initial reports on serious adverse events (hemoptysis) in patients primarily with squamous cell histology.(12) The efficacy of intracellular tyrosine kinase inhibitors of epidermal growth factor (EGFR) like erlotinib and gefitinib is also mainly found in adenocarcinoma. In our studies, bronchioalveolar carcinoma (BAC) has been mentioned as a subtype of adenocarcinoma due to its different prognostic significance. Other sub typing has not been elaborately covered in this report as there is no known prognostic or predictive impact of other histology subtypes.

2.1.3 Diagnosis and staging (TNM) and prognosis

There are currently no generally implemented screening programs for lung cancer. Recently, the first results from the randomized NCI-sponsored National Lung Screening Trial

(NLST) were published in NEJM.(13) US citizens between 55-74 years of age with a history of heavy smoking were randomly assigned to a yearly low-dose CT or regular chest radiography. Although the results are premature and there is a concern of overdiagnosis, there was significantly reduced lung cancer mortality in the CT screened population.

Since early lung cancers often are asymptomatic, the debuts of symptoms often indicate advanced disease. Today, a lung tumor is either an incidental finding on a chest x-ray/CT or a finding after dedicated investigation. The goal of the diagnostic procedures is to establish a sufficient evidence of the disease (histology) and clinical stage. A chest CT including the upper abdomen is usually required for a proper identification of a lung tumor and ruling out regional metastases in the chest as well as distant metastases in the liver and suprarenal glands. To establish a diagnosis, sampling of tumor tissue through tissue biopsy and/or brush cytology is usually obtained by bronchoscopy. However, peripheral tumors often require CT guided biopsy. After a confirmed diagnosis of NSCLC, further staging procedures are necessary to establish the extent of disease burden. Brain MRI and a bone scan are often done to rule out apparent brain and bone metastasis. PET scans are helpful and are increasingly becoming available to evaluate if there is mediastinal or distant metastasis and to define the tumor volume for treatment planning in radiotherapy. (14) Patients also undergo lung function tests to determine operability.

An updated TNM Classification of Malignant Tumors was published in 2009 and included a new classification for lung cancer.(15) The revised classification was a product of the International Association for the Study of Lung Cancer (IASLC) staging project (Figure 2). A correct TNM stage is pivotal to secure appropriate and correct therapy for the patients. For patients that are candidates for curative surgery, it estimates the postoperative survival and thereby guides the use of adjuvant therapy.

Table 2: The seventh edition of TNM classifications and stage groupings (adapted from ref 15)

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 subclassification description appears only once

2.1.4 Treatment of NSCLC

Surgery, chemotherapy and radiotherapy are the main pillars of NSCLC treatment. However, there is a current trend in lung cancer towards personalized treatment based on clinical factors, but more importantly on factors relating to the tumor tissue ("tissue is the issue").

2.1.4.1 Curable NSCLC

NSCLC is potentially curable with a > 50% chance of 5-year survival if no nodal metastases are present and the tumor is ≤ 5 cm (see Table 2 for survival details). Surgical resection is the treatment of choice in potentially curable patients, stage I-IIIA. Subsequent adjuvant chemotherapy has been introduced in NSCLC for stages IIA-IIIA. The patients in our studies were, however, treated prior to the introduction of adjuvant chemotherapy in NSCLC 2005.(16) Today, approximately 50 % have distant metastasis at presentation and a large number of diagnosed patients are inoperable due to comorbidity. This leads to the low overall resection rate of 20% recorded in Norway in 2008 with a promising increase recent years(17;18). Reduced lung function or serious heart conditions can lead to more marginal resections than otherwise wanted or even no surgery. A wedge resection is generally considered an inferior surgery for NSCLC, although a small non-randomized study has shown comparable results with standard lobectomy/bilobectomy or pulmonectomy.(19)

Postoperative radiotherapy in NSCLC should be given to patients with N2 disease and/or non-radical resection margins as these patients may have a survival benefit.(20;21) If patients present with locally advanced disease without poor prognostic factors (tumor size >8 cm, poor performance status and >10% weight loss last six months), they may be offered chemoradiotherapy. Medically inoperable patients may also be candidates for localized radiotherapy (60-70Gy) with or without chemotherapy.(22)

Today, there is no routine use of molecular targeted drugs in curative treatment of NSCLC. But there are a large number of trials of targeted drugs in adjuvant and even neoadjuvant setting for subgroups of NSCLC patients. Including vaccine studies, there are currently >30 studies with targeted drugs in clinical trials. (www.clinicaltrials.gov; accessed 15.02.11)

2.1.4.2 Advanced NSCLC

Advanced NSCLC, which constitutes the vast majority of patients, are treated with the aim of life prolongation and palliation. The benefit of chemotherapy in advanced NSCLC was debated as late as 1995 (23), but today a "platinum doublet" including platinum is considered standard and provides a survival benefit with a expected survival of 6-14 months. Overall quality-of-life (QOL) is also improved with chemotherapy, with improvements in disease-specific symptoms. (24) Furthermore, radiotherapy is an effective option, especially in reducing distressing symptoms like hemoptysis, cough, dyspnea and pain.(25)

Recently, molecular targeted therapies have been introduced in advanced NSCLC. Adding bevacizumab to standard first-line chemotherapy has improved response rates, but with minimal improvements in survival rates.(12) Drugs targeting the EGFR pathway have been studied in several NSCLC phase III trials. Monoclonal antibodies against EGFR, in the form of cetuximab, has been shown to marginally improve survival when added to chemotherapy in patients expressing EGFR (26), but has not been approved by the US Food and Drug Administration (FDA) or European medicines agencies. The small molecular tyrosine kinase inhibitors (TKI) of EGFR, erlotinib and gefitinib, have response rates of approximately 10% in an unselected population. Erlotinib has been approved by the regulatory authorities due to improved overall survival in second-line, whereas gefitinib has been approved only in patients with activating somatic mutations in the EGFR gene. Improved TKI efficacy has been shown in the following subgroups: Females, never smokers,

adenocarcinomas and East Asian ethnicity, most possibly representing a higher prevalence of activating somatic mutations in these groups, predicting response to EGFR therapy.(27) Guidelines depicting testing details for activating somatic mutations, interpretation and consequences of such testing are currently being developed in Norway and European consensus has also recently been met.(28)

2.2 Hypoxia and angiogenesis

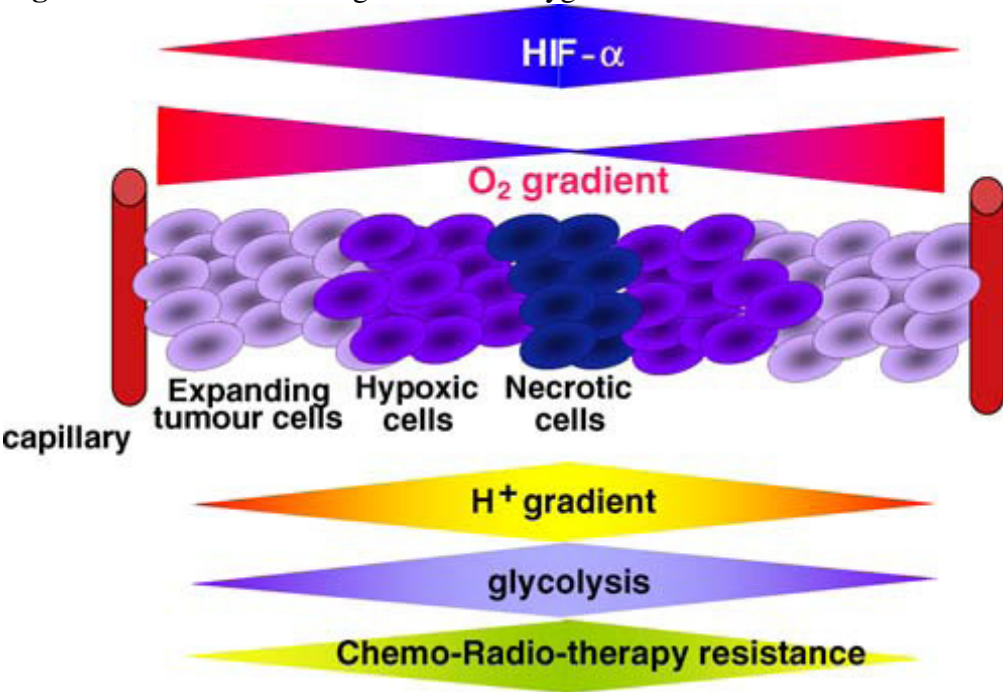
2.2.1 Hypoxia

Oxygen is a vital part of human metabolism as the presence of oxygen enables the cells to retrieve around 16 times more energy out of glucose than what is otherwise possible under anaerobic conditions. In growing tissue, as in tumors or during development, there is a changing oxygen pressure in which these cells have to adapt to survive and proliferate. Ambient air contains 21% O_2 (150 mm Hg). Most tissues are at 2-9% O_2, with tissue hypoxia usually defined at ≤2%. In lung tumors, median tumor oxygen tension has been measured at 2.2% (range 0.1-6%), indicating hypoxia to be a prevalent feature.(29). Based on histological studies of lung tumors, Thomlinson and Gray in 1955 proposed that the necrosis, found in cores surrounded by viable cells neighboring a capillary vessel, was due to hypoxia. (30) Later, hypoxia could be measured more precisely by electrodes, but there has been found a considerable inter- and intra-tumoral variability demanding more precise and dynamic scoring of hypoxia.(31;32) Additionally, this is probably even more complicated as hypoxia is not only a chronic feature, but can also be acute due to changing dynamics of blood flow. Furthermore, cancer cells with one type of hypoxia may have a different treatment response than cells with the other type.(33;34)

Hypoxia is a cardinal phenotype of the tumor environment, both due to tumor respiration and due to neoplastic cell colonization of tissues without a prerequisite blood supply. The low oxygen tension trigger the gene expression towards a more aggressive

phenotype and hypoxia reduces the sensitivity to therapy. These characteristics are the result of activation of key hallmarks of cancer like angiogenesis, metastasis, increased DNA replication and proliferation in both hypoxia induced factor (HIF) dependent and independent manners (see HIF section for details). The observation by Schwarz et al. that hypoxic cells are less sensitive to radiotherapy, is more than 100 years old.(35) Almost 50 years later Gray and others managed to establish evidence for the radioresistance seen in hypoxia in vitro.(30;36) In a following murine study, Powers and colleagues found decreased tumor cell survival in irradiated lymphosarcomas from mice breathing hyperbaric O_2, and Churchill-Davidson found evidence for the same phenomenon in cancer patients.(37;38) Although hypoxia is recognized as a key feature of resistance to radiotherapy, the later hypoxia-modifying studies with hyperbaric O_2 and hypoxic cell radiosensitizers, have in general been disappointing, thus hypoxic modification is normally not influencing clinical practice.(39) Hypoxia is also known to mediate resistance to chemotherapy, both directly and through the often simultaneously raised interstitial fluid pressure (IFP) (40;41)

Figure 3: Tumor cells and gradients of oxygen.



With a decreasing oxygen tension there is increased HIF1α activity, increased production of acid, increased rate of glycolysis and an increased resistance to chemo and radiotherapyFigure copied from ref (40). Permission obtaind from Springer©

2.2.2 Angiogenesis

Sustained angiogenesis was included as a hallmark of cancer in a renowned review by Hanahan and Weinberg.(42) Angiogenesis is the physiological phenomenon of growth of new vessels from pre-existing vessels as opposed to vasculogenesis where blood vessels are formed without pre-existing ones.(43) Oxygen and nutrients are brought to the tissues by diffusion and blood supply. Thomlinson and Gray measured the band of live cancer cells surrounding a vessel to be about 170 µm wide which is close to the calculated distance of O_2 diffusion 140 µm. If tumor cells are to survive and proliferate further away from vessels, new vessels will have to be made.(30) Folkman stated in 1971 that angiogenesis was crucial if tumors were to grow beyond 1-2 mm^3 and proposed the idea of targeting angiogenesis.(44) Angiogenesis is a complex and dynamic process. In physiological angiogenesis several factors are involved in a tightly regulated manner. Tumor angiogenesis is more chaotic, but orchestrated by the same factors.(45)

Some of the most important factors are growth factor families like vascular endothelial growth factor (VEGF), placental growth factor (PLGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and their receptors, as well as players in other pathways like the angiopoietins (Ang) and their Tie-2 receptor, NOTCH-Deltalike ligand 4 (DLL4) and endogenous angiogenesis inhibitors like vasohibin, angiostatin, endostatin, trombospondin-1 and tumstatin.(46)

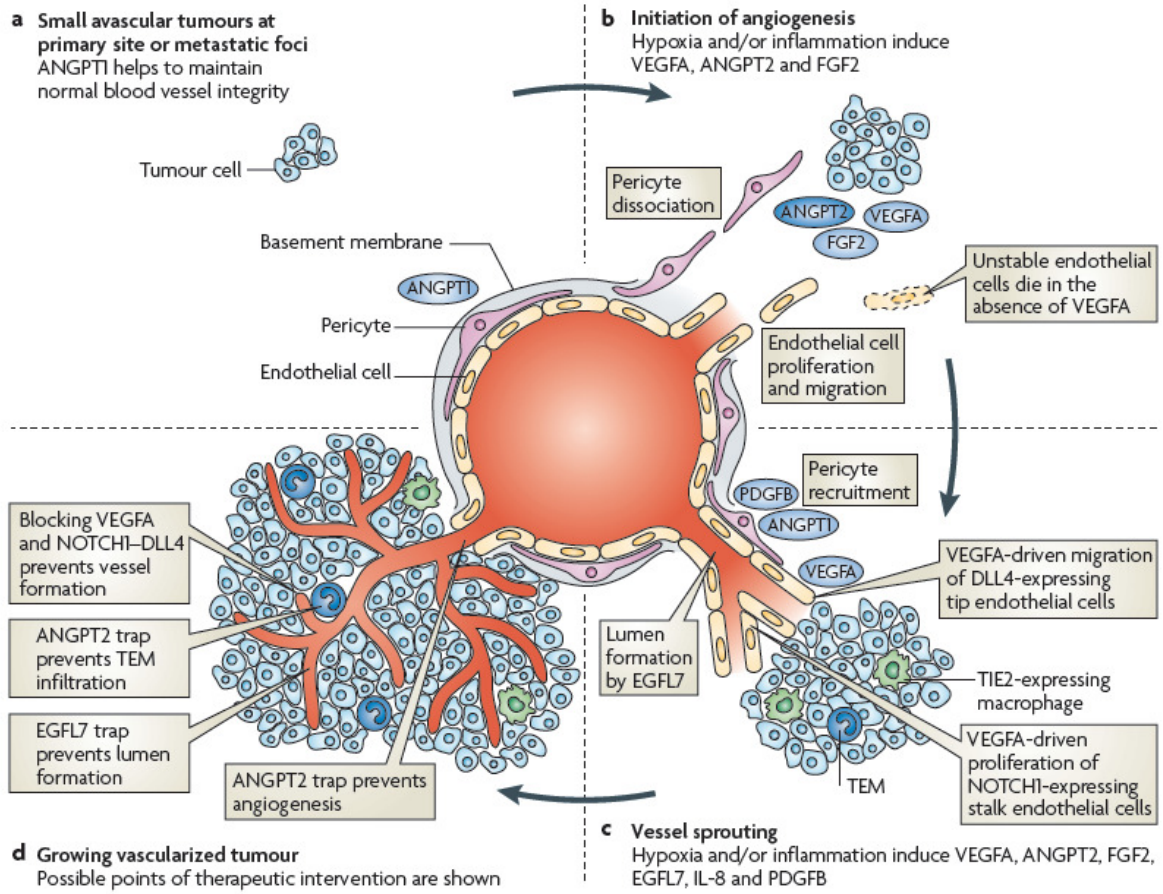


Figure 4: Angiogenesis with emphasis on angiopoietins.
a) A small focus of tumor cells served by diffusion. Angiogenesis not needed and pericytes stimulate endothelial cells (ECs) with Ang-1 to maintain vessel integrity and barrier function of ECs. **b)** Ang-2 mediates pericyte dissociation, increased permeability and sensitizes the ECs to growth factors, especially VEGF-A. **c)** Vessel sprouting is driven the migration of delta-like4 (DLL4) expressing tip endothelial cells following a VEGF-A concentration gradient. The proliferation of NOTCH1 expressing stalk ECs mediated by VEGF-A are elongating the sprout. These ECs are subsequently stimulated by Ang-1 secreting pericytes recruited partly by Ang-1 and PDGFB facilitating a robust vasculature. Epidermal growth factor-like domain multiple 7 (EGFL7) regulates lumen formation. **d)** A growing and vascularized tumor mass showing some of the possible therapeutic intervention points. As published in (47). Permission obtained from Nature Publishing Group©.

2.2.3 Biomarkers associated with hypoxia and angiogenesis covered in this thesis

2.2.3.1 Hypoxia induced factors (HIFs)

In 1992 Semenza and Wang recognized and purified the hypoxia inducible factor (HIF) as the promiscuous transcription factor at the heart of cell adaptation to shifting oxygen tension(48). HIF is the active heterodimer of HIFα and HIFβ. HIFα is one of the proteins with the shortest known half-life, but also detectable less than 2 minutes after exposure to

hypoxia.(49) The quick response of shifting oxygen tension facilitates rapid regulation of its gene targets. Stabilized HIFα will bind to the constitutive HIFβ-subunit and translocate to the nucleus. In the nucleus it will exert its action as a transcription factor, by binding to hypoxia responsive elements (HRE) in the promoter regions of target genes (Figure 5).

Among the three HIFα-subunits (HIF1α, HIF2α and HIF3α), HIF1α was the first HIF family member to be described and the most studied. HIF1α is ubiquitously expressed and induces a wide range of hypoxia-inducible genes. It is highly expressed in many different tumors, but infrequent in most normal tissues.(50;51) Over expression of HIF1α has consistently been found associated with a poor prognosis in a broad range of tumors (reviewed in ref (52)), also in NSCLC.(53) Also the HIF2α isoform seems important in physiology as targeted disruption of HIF2α leads to embryonic lethality. Although HIF1α and HIF2α share significant sequence homology, they have unique tissue distributions and effect on tumor progression. Whereas HIF1α is found almost in every tissue, HIF2α expression is more restricted and seems to be highly expressed in tissues mainly involved in systemic delivery of O_2, like lung, heart and endothelium. Regarding target genes, HIF1α uniquely stimulates the expression of many enzymes like lactate dehydrogenase 5 (LDH5) and carbonic anhydrase IX (CAIX), while transforming growth factor-α (TGF-α) and erythropoietin (EPO) are upregulated in hypoxia by HIF2α. Other transcriptional genes like glucose transporter 1 (*GLUT1)* and *VEGF-A* are commonly upregulated by both subunits.(reviewed in ref (54)) The contribution by HIF3α is not yet properly determined, but it is rapidly induced by hypoxia in most tissues, and a splicing variant appears to be an antagonist of the HIF system.(55;56)

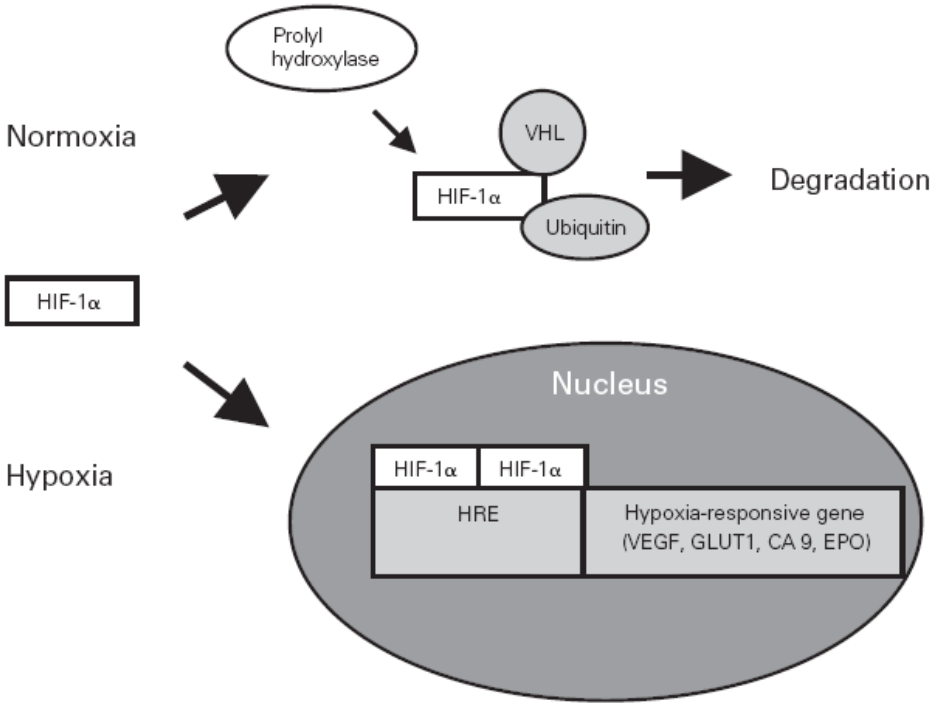


Figure 5: Schematic function of hypoxia-inducuble factor-1α.
In hypoxia HIF1α will be stabilized, translocated to the nucleus, dimerize with HIF1β, bind to a hypoxia responsive element (HRE) and lead to hypoxia-responsive gene activation. Under normoxia HIF1α will by hydroxylated by prolyl hydroxylases (PHDs) leading to von-hippel-Lindau (VHL) binding and ubiquitin mediated proteasomal degradation. As published in (57).Permission obtained from AACR©

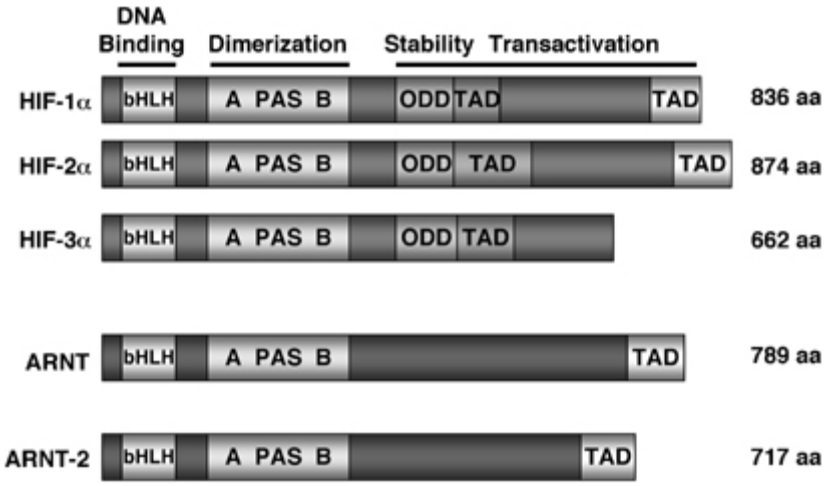


Figure 6: The Hypoxia induced factors (HIFs)-subunits.
The three HIFα subunits share structural similarities. The basic helix-loop-helix (bHLH) mediates DNA binding and the PER-ARNT-SIM (PAS) domain mediates dimerization of HIFα and HIFβ subunits. The potency of HIFs is related to the transactivation domains (TADs). An oxygen-dependent degradation domain (ODD) is the area in which hydroxylation due to prolyl hydroxylases occur, leading to proteasomal degradation. ARNT -1 and -2 are the isoforms of the HIFβ-subunit. As published in (52).Permission obtained from nature publishing group.©

2.2.3.2 HIF induced gene products

More than 100 well-defined gene products are induced by HIF.(58) These molecules can be grouped into several characteristic cancer cell qualities like altered:(59)

- Metabolism; GLUT1, hexokinase 1 and 2, LDH5
- Oxygen sensing; Prolyl hydroxylase (PHD) 2 and 3
- pH homeostasis; CAIX and CAXII
- Angiogenesis; VEGF-A, PLGF, PDGFB, Ang-2
- Erytropoiesis; Erythropoietin (EPO)
- Metastasis; Chemokine receptor type 4 (CXCR4), lysyl oxidase
- Invasion; Endothelin 1, fibronectin 1, matrix metalloproteinase (MMP) 2, 14 and C-met
- Genomic instability; Differentiated embryo-chondrocyte expressed gene 1 (DEC1)
- Cell survival /immortalization and autophagy; Survivin, telomerase and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3)
- Epithelial-mesenchymal transition (EMT); TWIST1, Zinc finger E-box-binding homeobox (ZEB) 1 and 2

In the following I will focus on GLUT1, CAIX and LDH5 since these are part of my IHC-studies. GLUT1 and CAIX were included due to their proposed, but controversial role as endogenous markers of hypoxia (60-62), and LDH5 because it is one of the most known HIF-induced molecules and involved in metabolism like GLUT1. Angiopoietins and prolyl hydroxylases are reviewed in other sections.

2.2.3.2.1 GLUT1

Sugars are an important substrate for energy production through cellular respiration with oxidative phosphorylation as the final and most productive step. However, cancer cells mainly utilize glycolysis as discovered by Warburg more than 50 years ago.(63) In fact, cancer cells

actually prefer glycolysis with or without the presence of oxygen. To achieve this phenotype, an increased import of sugars, mainly glucose, is needed. Other sugars like fructose can be used, but glucose uptake is the rate-limiting step. Sugars are hydrophilic and need to be transported into cells by glucose transporters. Transportation is mediated through membranes by membrane proteins such as facilitated glucose transporters (GLUTs) or sodium/glucose co-transporters (SGLT). There are several subtypes of these sugar transporters, but GLUT1 is responsible for the basal glucose uptake which is probably why it is the most studied glucose transporter in cancer. It is also related to the rate of glucose metabolism and it is expressed in all tissues. GLUT1 is induced by hypoxia (reviewed in ref (64)), but it is also known to be regulated by c-Myc.(65)

2.2.3.2.2 CAIX

Cellular biochemistry can be significantly altered by small changes in pH and proper regulation is vital for survival and function of cells. Cancer cells are characterized by a high metabolism and therefore must be able to handle the high intracellular production of excess protons (H^+) by transporting these from the inside of the cells to the extracellular environment. This can be achieved by Na^+/H^+-exchange, H^+-lactate co-transport or HCO_3^- (bicarbonate) dependent buffering with a subsequent extracellular CO_2 diffusion. For the bicarbonate-dependent buffering to be efficient, catalysis by carbonic anhydrases is imperative. In cancer, the extracellular bound carbonic anhydrase IX (CAIX) has been of increasing interest due to its induction by hypoxia, and that its expression has been found in many cancers, but rarely in normal tissues. Expression of CAIX is related to hypoxia below 1% O_2 and is therefore seen expressed between 80-130 μm from blood vessels (reviewed in ref (66)). Among the molecules related to hypoxia, CAIX has by some been proposed as one of the most reliable markers of hypoxia.(60;61), although this is still a matter of controversy.(62)

2.2.3.2.3 LDH5

Due to the high metabolic rate of cancer cells by glycolysis and the use of citric acid (TCA) intermediates for cancer cell membrane composition, there is a high production of pyruvate not needed for further energy production. Hence, pyruvate is converted to lactate by lactate dehydrogenases. Among five isoenzymes, lactate dehydrogenase 5 (LDH5), also called LDHA, has the highest efficiency in catalyzing pyruvate to lactate. Lactate can subsequently be transported to the extracellular space by a monocarboxylate transporter. LDH5 is also induced by HIF1α and is overexpressed in common cancers like NSCLC(67;68) head and neck cancers(69), non-Hodgkin B-cell lymphomas (70) and colorectal cancers (71).

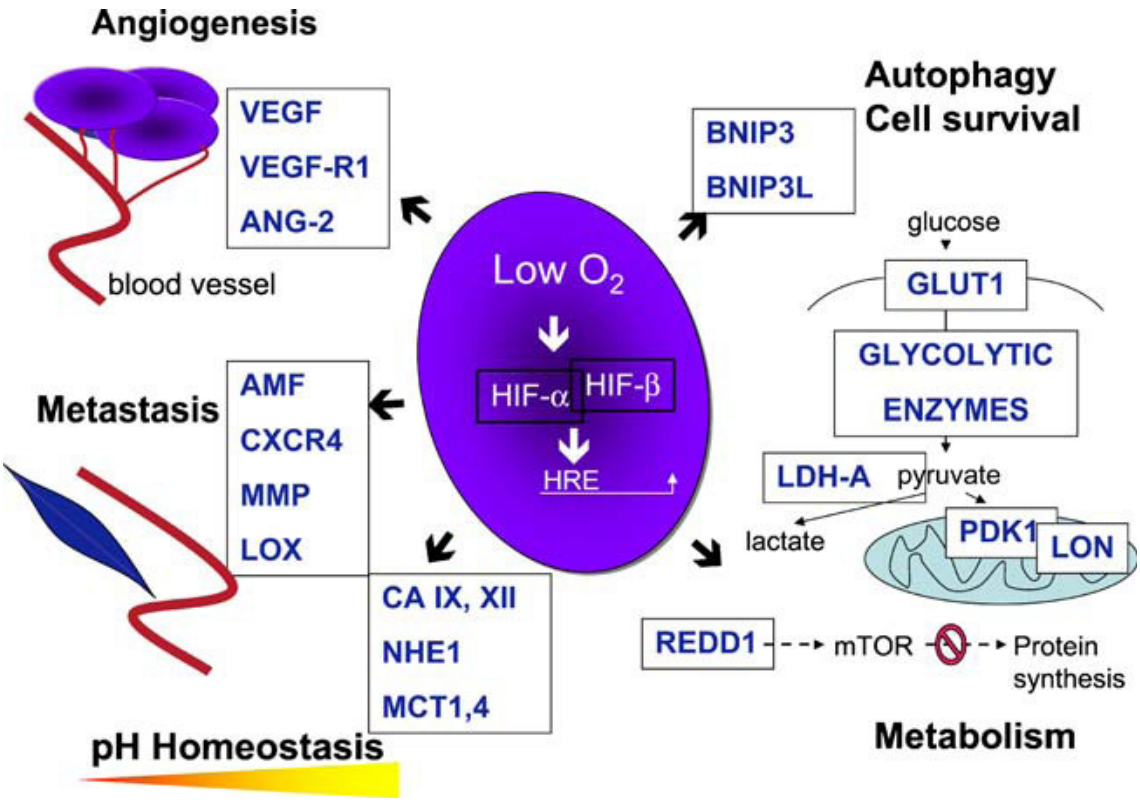


Figure 7: Examples of proteins grouped into several characteristic cancer cell qualities expressed as a result of a HIF-mediated hypoxia-response. As published in ref (40). Permission obtained from Springer©

2.2.3.3 Angiopoietins

Angiopoietin (Ang)/Tie-2 receptor signaling plays a critical role in concert with VEGF-A in angiogenesis.(72;73) This axis has proved crucial as withdrawal of VEGF-A

causes endothelial cells (ECs) lacking support of pericytes to undergo rapid apoptosis, while ECs, with supporting mural cells expressing Angs, survive.(72;74-76)

There are three known human ligands for the tyrosine kinase Tie-2, namely Ang-1, Ang-2 and Ang-4. Ang-1 stimulates the kinase activity of Tie-2 upon binding. Ang-2 has been shown to act as a context-dependent antagonist or agonist for Tie-2 with the antagonism as the best described effect.(77;78) Ang-4 is a ligand which seems to have the same agonistic effect on Tie-2 as Ang-1, but is less studied.(79;80) Tie-2 downstream signaling most importantly mediates cell survival which in the vascular compartment maintains vascular quiescence, but also exerts anti-inflammatory effects. There is also another tyrosine kinase receptor, Tie-1, which has no known ligands, but binds to Tie-2 and regulate its activity.(81) Although its function regarding angiogenesis has been mostly studied in ECs and pericytes, there are also accumulating evidence that expression of Ang-2 in tumor cells is involved in cancer progression and metastasis in glioma (82-84), gastric cancer (85), colorectal cancer (86;87), prostate cancer (88) and breast cancer (89).

2.2.3.4 HIF hydroxylases

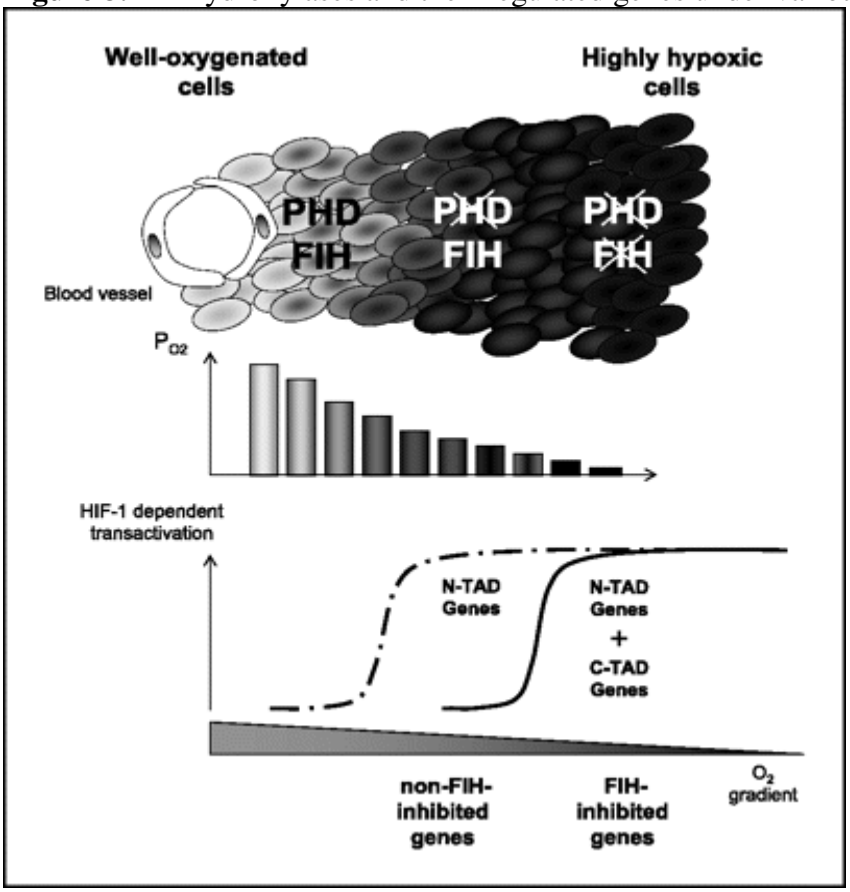
Regulation of the HIF activity is mainly controlled by the half-life of the HIFα-subunit, which is tightly controlled by the oxygen dependent post-translational hydroxylation by HIF hydroxylases. Under normoxia, HIFα is hydroxylated by prolyl hydroxylases (PHD1, PHD2 and PHD3) and factor inhibiting HIF (FIH). Hydroxylation of the proline residues in the N-terminal area transactivation domain (N-TAD) by PHDs enables binding with von Hippel-Lindau (VHL) tumor suppression protein with subsequent targeting of HIFα for proteosomal degradation by polyubiquitation.(90;91) The different PHDs share a 42-59% sequence similarity. Principally all PHDs have the same function, but appear to have different specificities for various hydroxylation sites.(92) PHD2 is the most abundant form and it is the main regulator of HIF1α activity, probably due to its relative abundance. RNA interference

against PHD2 induces stabilized HIF1α subunits whereas PHD1 and PHD3 silencing had no effect on HIF1α –stabilization. PHD3, on the other hand, more efficiently regulates HIF2α.(92;93)

FIH hydroxylates the C-terminal transactivation domain (C-TAD) of HIFα. This prevents transcriptional activation due to the inhibitory effect of this hydroxylation on the interaction between HIF and coactivators like p300.

Together, these hydroxylases are known as HIF hydroxylases and serve the function of oxygen sensing in the vital cellular oxygen homeostasis.(94;95) PHD expression has been detected in most normal human tissues, but in malignancy PHDs are overexpressed in common cancers like breast, prostate and head and neck whereas a slight decreased expression compared to normal tissue was suggested in lung cancer, renal cell carcinomas and follicular lymphomas (reviewed in ref (96)).

Figure 8:HIF hydroxylases and their regulated genes under various oxygen tension



HIF1α has two transactivation domains (TADs); a N-terminal domain (N-TAD) and a C-terminal domain (C-TAD). With decreasing O_2-tensions the hydroxylation by the HIF-hydroxylases (PHDs and FIH) are decreased, inducing stabilized HIF1α with transcriptional activity. However, PHDs require higher oxygen tension than FIH for hydroxylation. This enables a two-step regulation of HIF1α. At moderate hypoxia, PHD function is terminated resulting in stabilized HIF1α which can transcribe N-TAD genes, but transcription of C-TAD genes is still blocked by the hydroxylation of FIH. In severe hypoxia, both FIH and PHDs are inactive enabling transcription of both N-TAD and C-TAD genes. As published in ref (57). Permission obtained from AACR©

2.3 Predictive or prognostic biomarkers

A biomarker is defined according to the following: It must be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a specified therapeutic intervention.(97) It must also harbor properties valuable in the clinical setting, either as a prognostic or predictive marker.

A prognostic marker is a factor showing a statistically significant association between its presence and outcome. A clinical useful prognostic marker must also be statistically independent, easy to determine and interpret and have therapeutic consequences. Prognostic

biomarkers for progression, relapse and survival are important for patient handling and treatment, and especially in the patient selection for adjuvant treatment strategies.

A predictive marker is a factor showing a statistically significant association with the benefit from being assigned to a specific therapy. A biomarker with predictive value gives information on the effect of a therapeutic intervention in a patient. It is therefore helpful in identifying subgroups of patients with differential responses across therapies.(98)

2.4 Implemented biomarkers and clinical outcome in NSCLC

Molecular marker research has exploded during the last decade, also in NSCLC. But few have, hitherto, adopted into current clinical practice. Molecular markers must harbor properties that are valuable in the clinical setting, either as prognostic or predictive markers. These have been reviewed in ref (99-101).

Epidermal growth factor receptor (EGFR) mutations: EGFR (also named HER1) is one of four epidermal growth factors. As more than 60% of NSCLC patients express EGFR, EGFR has become an important therapeutic target for treatment in NSCLC. Initial subgroup analyses identified clinicopathological factors like Asian ethnicity, female sex, adenocarcinoma histology and light/never smokers to predict response to EGFR TKIs. However, we are now aware that these factors select for a population with sensitizing mutations within the TKI domain, which 10-15% of patients have. Around 85-90% of known EGFR activating mutations are either exon 19 microdeletions or exon 21 point mutations. (102;103) Recently, promising, but premature results from the EURTAC were presented at ASCO 2011 showing 55% response rate to erlotinib for patients with activating mutations vs 11% for patients with activating mutations randomized to chemotherapy.(104) In a up-to-date review Ellis et al. conclude that diagnostic lung cancer samples of patients with advanced

NSCLC of non-squamous histology should be routinely tested for activating mutations in the first-line setting.

Echinoderm microtubule-associated protein-like 4 (EML4) and the anaplastic lymphoma kinase (ALK) fusion (EML4-ALK): A translocation of these genes results in a fusion protein resulting in a distinct entity of NSCLC. There is no specific therapy approved for ALK-associated NSCLC, but as targeted agents are emerging I have included this biomarker although not implemented yet. The EML4-ALK translocation is found in as few as 1-7% of NSCLC patients. This translocation seems to serve as a predictive marker for ALK inhibitor therapy. These translocations and EGFR mutations are mutually exclusive. (101;105)

No prognostic molecular biomarkers have been generally implemented although several have been suggested.

2.5 Microenvironment - tumor vs stroma

Although the "seed and soil" hypothesis was presented more than a century ago by Stephen Paget we are now starting to comprehend the complex crosstalk between the tumor cells (the "seeds") and the tumor-growing microenvironment (the "soil"). Tumors are complex tissues composed of malignant neoplastic cells as well as a tumor stroma with various non-malignant cells and extracellular matrix (ECM). Stromal properties can be defensive, permissive or even cooperative towards malignant cells to facilitate growth, invasion and metastasis. The malignant tumor has been compared to a new organ within the tissues as malignant cells are able to recruit and exploit the microenvironment to produce a supportive microenvironment for the neoplastic cells.(106-109)

The ECM contributes to the stroma with structural proteins (collagen, elastin), specialized proteins (fibrilin, fibronectin, elastin) and proteoglycans. NSCLC tumors are also

a heterogeneous mixture of cells. The main cells in a NSCLC tumor are the neoplastic cells, mesenchymal supporting cells like fibroblasts and adipocytes as well as vascular cells and immune cells. I will here present fibroblasts and immune cells.

2.5.1 Carcinoma associated fibroblasts

Fibroblasts are activated by tissue injury with the function of depositing and turning over ECM. In tumors, activated fibroblasts constitute a major portion of the reactive tumor stroma and play a crucial role in tumor progression. Due to the acknowledgement of their supportive role in tumor progression they have been entitled carcinoma-associated fibroblasts (CAFs). They have, however, been hard to identify due to a lack of a cell-specific marker and are defined for the most part of what they are not, non-smooth muscle cells, non-endothelial cells and non-epithelial cells. However, during activation many of them express smooth muscle actin and these are referred to as myofibroblasts or activated fibroblasts. By secretion of growth factors, ECM-degrading proteases, involvement in endothelial-mesenchymal transition (EMT) by e.g. TGFβ they are involved in cancer promoting features like growth, migration and invasion. Their role in cancer have been extensively reviewed (107;110), and the role in NSCLC has recently been reviewed by our research group.(111)

2.5.2 Immune cells

The immune system in cancer has also been described to be involved in both cancer-protective and cancer-promoting features. However, in already established malignancies, there is accumulating evidence for the role of the immune systems in tumor progression, invasion, metastasis and avoidance of an effective immune response.(112-114) The immune system can be separated in two distinct systems: The innate and adaptive immune system.

The innate immune system involves cells as dendritic cells (DCs), natural killer (NK) cells, macrophages, mast cells and granulocytes. These are our first defense line against foreign pathogens and they are able to react rapidly to tissue injury. Normally, an innate

immune response is temporary and tissue homeostasis is restored thereafter. However, in cancer there is chronic inflammation and several subsets of the innate immune system have been shown to exert tumor promoting effects. For instance, tumor-associated macrophages (TAMs) are mostly activated through the M2 pathway known to promote invasion, angiogenesis, metastasis and immunosuppression in carcinomas and when found in tumors, they have consistently been associated with a poor prognosis.(112-114)

The cells in the adaptive immune system are B-lymphocytes which are mainly antibody producing cells and T-lymphocytes of CD4+ (helper) or CD8+ (cytotoxic) subtypes. These cells are specialized by a slower, but a sophisticated and effective antigen-specific response. In cancer, the role of the adaptive immune cells is debated. In favor of tumor-promotion are the classic regulatory T cells which are CD4+CD25+FOXP3+. These cells suppress the otherwise effective anti-tumor responses of cytotoxic T-cells. Depending on the etiology of the specific microenvironment the adaptive immune system can be tumor-promoting or suppressing.(112-114)

3 AIMS OF THESIS

Based on existing knowledge about angiogenesis, hypoxia and stroma described in chapter 2, the general aim of this study is to study known biomarkers involved in angiogenesis and hypoxia with regards to their relevance in NSCLC progression.

More specifically the aims of this thesis are to:

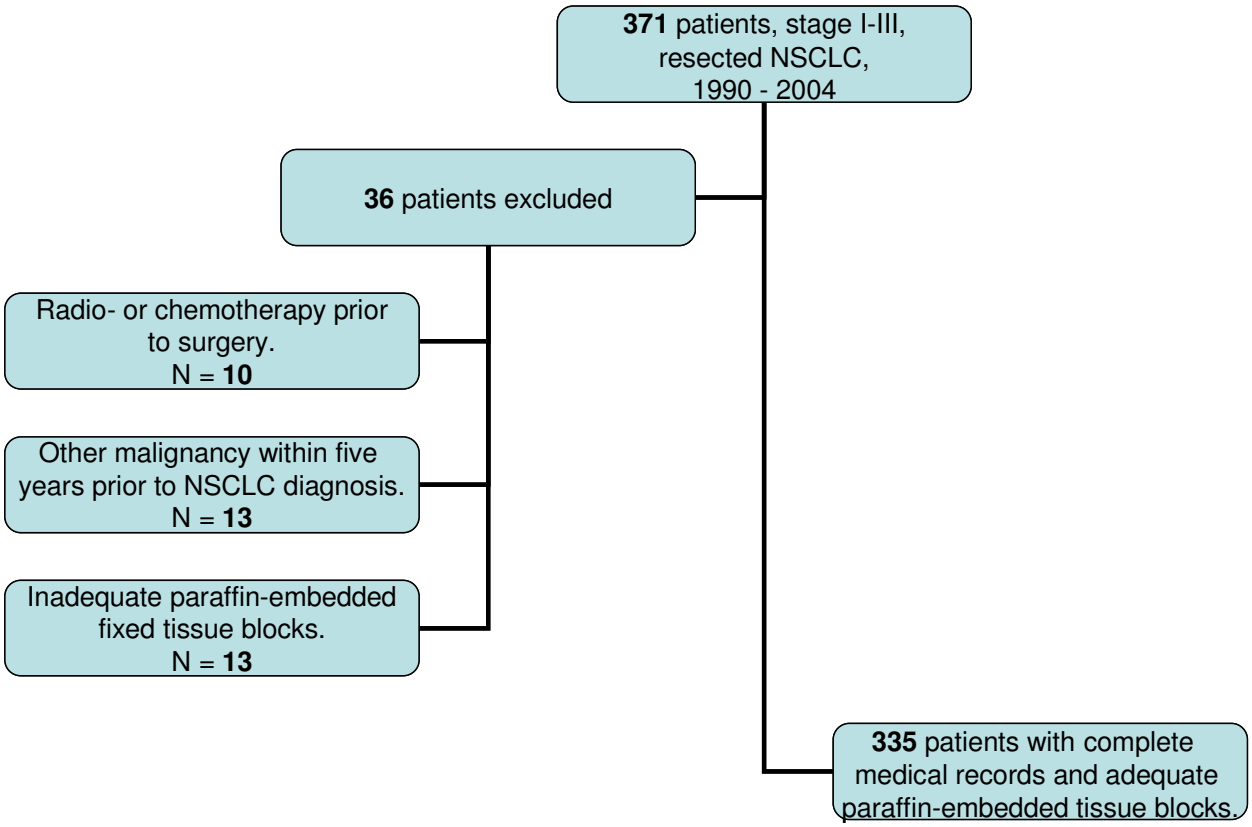
1. Examine immunohistochemical expression of important molecules in angiogenesis and cell adaptation to hypoxia in NSCLC
2. Examine the prognostic impact of these biomarkers for disease-specific survival alone and adjusted for known prognostic factors
3. Help establish candidate markers for better prognostication for NSCLC patients
4. Suggest possible targets for therapy

4 MATERIALS AND METHODS

4.1 Patients

Patients with pathological stage I to IIIA NSCLC were identified through the pathological departments at the University Hospital of Northern Norway (UNN) in Tromso and the Nordland Central Hospital in Bodo. Among the 371 identified patients, 36 were excluded from the study due to: Radiotherapy or chemotherapy prior to surgery, other malignancy within five years prior to NSCLC diagnosis, or inadequate paraffin-embedded tissue blocks (Figure 9). Patient characteristics are presented in Table 6, section 5.1.

Figure 9: Patient inclusion and exclusion



4.2 Tissue Micro Array

Tissue microarrays have revolutionized molecular profiling in cancer as it has enabled high-throughput analysis. The first published report which can be associated to TMAs was by Battifora et al. who in 1986 described the multitumor “sausage” tissue block.(115) However, the first study describing a modern technology of tissue micro array (TMA) was by Wan et al. in 1987(116), although the casing for the cores were cut from ordinary drinking straws! Ten years later Kononen et al. (117) published on the development a device for rapid production with high quality and is referred to as the first modern TMA study.

The most common form is the one were you take a tissue core biopsy (0.6-2 mm diameter) from a preselected area of interest in the tissue and insert it in a predrilled paraffin recipient block where a grid system makes it easy to link it to the patients’ clinicopathological data. Immunohistochemistry is the usual assay method, but other in-situ techniques like fluorescent in situ hybridization (FISH) have been used. More advanced explored alternatives are ultrahigh density microarrays where solid samples are stacked together, (118) use of frozen tissues,(119) use of cells from suspension (120) and some have even used needle biopsies as tissue basis.(121)

The advantages of the TMA technology are clearly demonstrated if you consider the alternative with many whole sections. This requires the use of different batches requiring quantities of reagents and thorough manual examination of each slide. TMAs save time for technicians and pathologists, speeds up time from data collection to publication, specimens are stained at the same time and conditions securing standardization, tumor scoring can be done reliably by non-specialists, the tissue is saved as only small amounts of each tissue is needed to produce the TMA, a larger number of patients can be included which increases quality and power of the study and lastly the TMAs can be easily shared across research groups making validation easier.(122) Advantages and disadvantages of TMAs are listed in

Table 3. In the following sections I will go through some of the processes and how we have addressed these to ensure quality.

Table 3: Advantages and disadvantages of tissue microarray (TMA) technology.

Advantages (+)	**Disadvantages (-)**
Saves time for technician and pathologist	Reduced representativity for heterogeneous stained tissue
Tumor scoring can be done by non-specialists	Not suitable for diagnosis
Cost saving	Dependent on TMA producing skills
Specimens are stained consistently	Still dependent on the quality of patient cohort
Saves tissue	Still dependent on antibody quality
Can be shared	Still dependent on the quality of tissue
Ability to assay a large number of tissues	

4.2.1 Tissue sample acquisition

Under the surgical procedure, degradation of various molecules starts as soon as the surgeon cuts blood supply to the tumor. The time from loss of blood supply to the tissue arrival at the pathological department can vary leading to a difference in expression of several proteins. This can theoretically be countered by standardized and rigorous tissue banking regimens, but there is also evidence that for instance RNA degradation occurs during surgery and that post-surgery banking regimens can not compensate fully.

Our tissues have been collected from routine specimens which have been exposed to varying time spans without blood supply before arriving at the pathological departments, and we have no reliable way to reproduce the accurate time factor. However, it has been stated that the most valuable tissue samples with long follow-up are stored in routine pathological archives and that standardization of tissue handling might be too demanding considering the modest benefit of a stringent regimen.(123)

4.2.2 Tissue sample preparation

Another important factor of post-surgery tissue handling is fixation techniques. The techniques may have changed during the time span the patients were treated, both regarding reagents and pathological preparation. Since penetration time of formalin is estimated to about 1 mm an hour,(124) tissues of different sizes will lead to varying time for actual protein cross-linking. We have like many others used archival tissues from a considerable time span, permitting variations in tissue processing techniques to bias our results. But there has been no systematic change in tissue handling that may systematically have bias our results. All nine TMA-slides had the same delay between cutting and staining. This is important as this delay has great influence on IHC-intensity.(125)

4.2.3 Histological examination

To include cases in our TMA-study we screened the pathology departments' databases for suitable cases. However, diagnostic criteria for the actual diagnosis and TNM classifications may change over time. In addition, routine specimens may be handled by different pathologists. To address this issue our two experienced pathologists re-examined the diagnosis and revised the TNM-status as well as factors like vascular infiltration and differentiation for all of the included patients. In addition they selected the most representative areas of both tumor and stroma for sampling by our experienced technician.

4.2.4 Collection of clinical data

The ambition of most TMA-studies in cancer is to find associations between clinical outcome versus biomarker(s), and/or clinicopathological variables. Outcome and clinical data has to be collected from archived medical journals, possibly at different geographical locations where the patient has received clinical follow-up. Age and gender are variables that can be easily collected. Other variables included in our study, like smoking status, performance status and weight loss, are regularly lacking in the immediate pre-surgery

examination, leading to missing data if not a thorough examination by individuals with medical experience and medical record interpretation is done. In our case, the three individuals responsible for collection of data were all experienced oncologists. Updates with regards to outcome have been done.

The low number of private institutions (none in our region) in Norway and the low level of geographical migration within Norway of these patients make it easier to collect reliable and comprehensive follow-up data.

Regarding clinical outcome data, several endpoints can be selected. As our follow-up is long and the most lung cancer patients relapse early, we were able to use disease-specific survival (DSS) as the primary endpoint as the numbers of events were sufficient (137 lung cancer deaths, 40.9% of patients). Regarding cause of death, it may be difficult to determine the cause exactly. Where uncertain, we have for practical reasons assigned patients to lung cancer death if they died with therapy resistant local or metastatic progression of disease.

4.2.5 TMA assembly

After review of the patient tissues, the most representative blocks were chosen. The most representative areas of tumor and stroma were then selected for tissue sampling with a 0.6 diameter tissue-arraying instrument from Beecher Instruments, Silver Springs, MD. This thin-walled stainless steel biopsy needle mediated the sampling of cylindrical samples from donor blocks and the extrusion of this core into the predrilled recipient block. Two separate predefined tumor areas and two predefined stromal areas were sampled from each tumor. A total of 335 x 4 = 1340 cores were sampled and inserted into 8 recipient blocks. In addition 20 control specimens were collected in the same way from normal lungs and inserted in an additional control TMA-block. For the IHC- analyses, 4 μm sections were cut with the Micron microtome (HM355S).

4.2.6 Representativity

Heterogeneity is a known feature in tumors (genetic, cell origin, protein expression, etc). In our study the mean NSCLC tumor size is 44 mm in maximum diameter. If we assume the tumors to have a perfect spherical form they have a mean volume of 9.21 cm^3. Our TMA cores have a volume of 0.00000011 cm^3, thereby visualizing 1/84 000 000 of the tumor. We employed duplicate cores in our study which increases the part of tumor or stroma visualized to 1/42 000 000. However, these numbers and the concern of representativity need to be put into context. A whole tissue section would only visualize 0.00024 cm^3, which would equal 1/38 000 of the same tumor. Considering these numbers it is easy to see that both a TMA core and a whole section can be questioned with regards to representativity. The few studies comparing large section analysis and TMA analysis have found them to be equally relevant for predicting prognosis of histological grade and Ki-67 in bladder cancer, ER and PR in breast cancer while TMA actually was found to be superior in predicting prognosis for p53 expression.(126;127)

Another way to increase representativity is to increase the number of cores from each tumor. This also increases workload, but is more advantageous than increasing core size.(128) This is especially important in studies with small patient cohorts. Larger cores can be justified as an alternative if more normal tissues are to be evaluated since one would need larger diameter cores to include all of the relevant cells. In our study this would have been advantageous for stromal sections as larger cores could have made us able to evaluate vessels and vessel expression of markers in stromal tissues.

4.3 Immunohistochemistry (IHC)

Immunohistochemistry (IHC) is only one way of determining the expression of a given biological molecule, but it is widely adopted and it is considered the gold standard for *in-situ* protein expression evaluation in tissue sections. Immunohistochemistry has the advantage of being relatively inexpensive, it is established in most laboratories, staining intensity can be evaluated with little training, it can be done on archival tissues and expression can be evaluated *in-situ* to assess cells in different compartments and of different origins. In addition it is the final protein available in the tissues that is responsible for the effect of the marker, not the mRNA.

For the commercial antibodies the manufacturers often provide recommendations for IHC-protocols for their antibody and for non-commercial antibodies the scientists often provide a protocol or a reference. However, these protocols have to be adapted in the local laboratory. There is no standardized, commercially available equipment for antigen retrieval so although the principles are the same, a wide variety of adaptations have to be made to find the best set-up for antigen retrieval, incubation time of antibodies, dilutions, washing time and techniques. You are therefore highly reliant on experienced technicians and thorough evaluation of the results from different protocols before doing the actual staining of the TMA-tissues. Often, such rigorous demands means different antibodies have to be tested and new batches ordered before a satisfactory result is obtained. Occasionally the test of a potential biomarker has to be abandoned due to unsatisfying results. In our study, evaluation of HIF3α expression was abandoned due to unsatisfying staining with available antibodies.

In our lab, two very experienced technicians have done all of the IHC-experiments. After satisfying staining is achieved, minimal background and expected *in-situ* staining, the slides are evaluated by an experienced pathologist with regards to specificity, controlled against either available literature and/or positive and negative tissue controls.

4.3.1 Antibodies

The antibody is the pivotal reagent common to all immunohistochemistry. During the last 10 years we have seen an enormous expansion in the number of available antibodies directed towards an expanding number of antigens. There are two principally different subgroups of antibodies according to clonality; polyclonal antibodies and monoclonal antibodies.

Polyclonal antibodies are a heterogeneous mixture of antibodies directed against one antigen, but different epitopes. The antibodies have been generated by different B-cell clones of an animal and the antibodies have slightly different specificities and affinities to the antigen. Polyclonal antibodies are collected from the serum of previously immunized animals by injection of an antigen/immunogen. The typical animal is a rabbit.

Monoclonal antibodies are homogenous antibodies directed against one epitope of the antigen by production from a single B-cell clone. To achieve this, the spleen is taken out of a sacrificed immunized animal and the B-lymphocytes are isolated. These cells are then fused with immortalized myeloma cells. These new cell lines are further cultivated to identify and select the best cell line clone. You can enable this new isolated cell line to produce antibodies either in an artificial system (bioreactor) or you can inject these cells into the peritoneal cavity of an animal.

Regarding advantages and disadvantages of monoclonal or polyclonal antibodies there are benefits and drawbacks with both. Polyclonal antibodies are more robust in antigen binding due to their ability to identify several epitopes of the antigen. The tissue specimens can, due to tissue sample preparations, conceal the specific epitope which a monoclonal is directed against. Consequently, false negative IHC results with polyclonals are infrequent. However, specificity is a concern since the chance of cross-reactivity of these polyclonal antibodies is increased making a false positive result more likely. Monoclonal antibodies also

have a superior lot-to lot consistency since antibody production is not dependent on the life of the specific animal due to the immortalized cell line. Reviewed in ref (129)

Antibody generation is understandably a costly and time-consuming task. Therefore most researchers purchase commercially available antibodies. Antibody selection is a critical step in performing a reliable IHC-study.

The antibodies in our study were carefully selected by reviewing available literature. We selected antibodies which seemed to have worked well with others or by the information provided by the manufacturer. It was a prerequisite that the applied commercial antibodies had been subjected to in-house validation by the manufacturer for IHC on paraffin-embedded material (IHC-P). One exception was Ang-1 which was selected due to other researchers' published success with this antibody (130-134) and failure to achieve satisfying quality with other available IHC-P tested antibodies. The antibodies provided by The Department of Clinical Laboratory Sciences, University of Oxford, and Cancer Research UK, Molecular Oncology Laboratory, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK had previously been tested for IHC on paraffin-embedded material.Antibodies used in the studies are listed in Table 4

Table 4: Antibodies used in the studies

Antigen	Type	Manu-facturer	Catalog #	Dilution	Procedure	Antibody incubation	Antigen retrieval
HIF1α	Mouse Monoclonal	Novus Biologicals	NB100-131	1:3500	Ventana	overnight at 4°C	CC1 mild (heat treatment,30 min, Tris-based buffer)
HIF2α	Rabbit Polyclonal	Abcam	Ab199	1:40	Ventana	overnight at 4°C	CC1 mild (heat treatment,30 min, Tris-based buffer)
LDH5	Rabbit Polyclonal	Abcam	Ab53010	1:100	Manual	overnight at 4°C	Microwave Citrate-buffer pH 6
GLUT1	Mouse Monoclonal	Abcam	AB40084	1:500	Ventana	overnight at 4°C	CC1 standard (heat treatment,60 min, Tris-based buffer)
CAIX	Rabbit Polyclonal	Abcam	Ab15086	1:200	Ventana	overnight at 4°C	CC1 mild (heat treatment,30 min, Tris-based buffer)
Ang-1	Goat Polyclonal	Santa Cruz	sc-6319	1:100	Manual	overnight at 4°C	Microwave Citrate-buffer pH 6
Ang-2	Rabbit Polyclonal	Abcam	Ab65835	1:30	Ventana	overnight at 4°C	CC1 mild, (heat treatment,30 min, Tris-based buffer)
Ang-4	Goat Polyclonal	R&D Systems	AF964	1:50	Manual	overnight at 4°C	Microwave Citrate-buffer pH 6
Tie-2	Rabbit Polyclonal	Santa Cruz	sc-9026	1:50	Ventana	overnight at 4°C	CC1 extended (heat treatment,90 min, Tris-based buffer)
VEGF-A	Rabbit Polyclonal	Neomarker	RB-1678	1:10	Manual	30 min at room temperature	Microwave Citrate-buffer pH 6
PHD1	Mouse Monoclonal	Abcam	Ab82884	1:50	Ventana	overnight at 4°C	CC1 mild, (heat treatment,30 min, Tris-based buffer)
PHD2	Mouse Monoclonal	Donated by *	366G/76	Undiluted	Manual	overnight at room temperature	Microwave Citrate-buffer pH 6
PHD3	Mouse Monoclonal	Donated by *	EG188e	Undiluted	Manual	overnight at room temperature	Microwave Tris/Edta buffer pH 9
FIH	Mouse Monoclonal	Donated by *	162c/D6	1:5	Manual	30 min at room temperature	Microwave Citrate-buffer pH 6

* Department of Clinical Laboratory Sciences, University of Oxford, and Cancer Research UK Molecular Oncology Laboratory, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK

4.3.2 IHC procedure

The nine 4 μm TMA sections containing the tissue cores were deparaffinized with xylene and rehydrated through graded ethanol series (manual staining) before antigen retrieval (described in Table 5). Antigen retrieval was for the automated procedures done by the Ventana Benchmark, XT automated slide stainer (Ventana Medical systems, Illrich, France).

The visible and evaluable staining requires a multistep procedure involving the primary antibody bound to the targeted protein, an enzyme complex and a chromogenic substrate.

The staining was a multistep procedure, involving a primary antibody which binds to the target, and a detection reagent containing a secondary antibody conjugated with an Avidin/Biotin/enzyme complex (manual procedure), or a polymer of secondary antibodies conjugated with an enzyme (Ventana procedure). In the end a chromogenic substrate was added, yielding a brown/reddish color at the site of the enzyme (both manual and automated procedure). The manual detection reagent used was “ABC Elite” kit from Vector labs. The detection system was the Ventana XT is Ultra View DAB. After visualizing the antibody target, the slides were counterstained with Hematoxylin to visualize the nuclei.

4.3.3 IHC controls

Positive and negative controls are required in many biological experiments to ensure adequate experimental quality. For immunohistochemistry, various control measurements can be implemented. To ensure that the antibody is specific, a western blot to ensure that binding of a protein with expected size is done. This procedure has usually already been performed by renowned and stringent manufacturers, and we have trusted these. In a recent publication, however, it has been argued that the purchaser should validate the specific binding.(135)

In the actual immunohistochemistry-procedures, negative and positive controls are used. This is principally done by including a tissue which does or does not express the

antigen. This tissue should be stained with the test tissue. Often the actual test tissue itself has a known expression with both negative and positive cells. However, if one wants to perform research on novel molecules which have not previously been tested in large IHC-studies, one has to compromise on these demands, but with increased suspicion regarding staining.

For the actual IHC procedure, negative reagent controls by replacing the primary antibody with a primary antibody diluent to check that there is no staining without the antibody. The negative control can be more rigorous by doing an isotype control. Isotype control antibodies are used to estimate the non-specific binding of target primary antibodies due to Fc receptor binding or other protein-protein interactions. An isotype control antibody should have the same immunoglobulin type and be used at the same concentration as the test antibody. Another control, although less used, is the pre-absorption control experiment where excess amounts of target protein (if available) are premixed with the primary antibody before incubation with the tissue. As all antibodies should be pre-bound to the available antigen in the solution, no staining should be detected. Some recommend this control especially for polyclonal antibodies as some clones may be non-specific.(136)

The positive IHC controls are to ensure that tissue supposed to be stained is actually stained. This is mostly done by including human tissue in the IHC-procedure which is consistently known be positively stained. Some have even constructed TMAs of various cell lines with known expression levels for validation. Some of these cell lines can even be formalin fixed cells transfected with the target protein. The tissue itself can also be the positive control if previous staining patterns for the tissue are known. By including stroma in the TMA studies a lot of non-neoplastic cells are included for which an expression profile is usually established. This aids validation of staining as we can control our staining against established expression profiles.

In our experiments we have used the following controls:

Paper 1: Negative controls were simultaneously performed for all antibodies by omitting the primary antibody. No formal positive controls as the target proteins had well known expression in NSCLC

Paper 2: Negative controls were simultaneously done for all antibodies by omitting the primary antibody, and an appropriate isotype control was done for all antibodies on one of the TMA slides. Capillary vessels in stromal cores with high expression were used for internal positive controls and skin hemangiomas as external positive controls.

Paper 3: Negative controls were simultaneously performed for all antibodies by omitting the primary antibody. For the commercial antibody for PHD1 we used normal testis as a positive control. For the donated antibodies, staining patterns in tumor and normal lung were compared to what has been published earlier regarding staining with these antibodies in NSCLC and lung tissues, to ensure the proper staining with these antibodies.[13,14] Validation on transfected cell lines with positive and negative controls has previously been published by others.[13,14]

4.4 Microscopic evaluation of staining

Manual evaluation of staining is time consuming and semi-quantitative. Automated systems have been applied and are commercially available. But such systems render separate assessment of subcellular staining difficult, e.g. cytoplasmic vs nuclear. They also exclusively score staining intensity of the whole core which makes scoring of distinct tissue compartments impossible, e.g. tumor vs stroma. From the beginning we aspired to use commercially available automated equipment, but the results were not satisfying. A major drawback was that the machine could not reliably distinguish tumor from stroma and as many cores in our TMA contain a mix of neoplastic cells and stromal cells we were obliged to use

manual scoring. To minimize subjectivity all cores were scored by two trained pathologists. Before initiating scoring we reviewed the staining with controls again for quality reassurance, we agreed on what compartments to score, we agreed on the semiquantitative scale and we agreed on illustrative examples of the different scores. In IHC studies several scoring scales have been utilized. Every scoring system should have stratification for no expression and high expression as a potential clinical significance will show statistical significance between these two subgroups. Several scoring systems score the percentage of positive tumor cells as well and calculate a combined score of intensity and percentage of positive cells(136), like the Allred score(137) and the H-score developed by McCarthy.(138) Both for GLUT1 and CAIX a combined score could be meaningfully calculated due to heterogeneity in expression within the tumor. However, this combined score did not result in any significant change of our results. A simple intensity score was therefore used for all published results.

Each anonymized core was scored independently, semiquantitatively and simultaneously by our pathologists by light microscopy. Only viable parts were scored and even though many cores comprised of both tumor and tumor stroma, only one entity was scored at a time. In stroma, we scored all non-neoplastic cells as a whole and did not differ between cells of different origins.

For all of our antibodies there was a multilevel intensity of staining. If possible we tried to score the dominant intensity in tumor and stroma according to the four level scale; 0 = negative, 1 = weak, 2 = intermediate and 3 = strong. The intensity was scored in the cell compartment with significant scorable expression and where expression was anticipated according to previous publications and/or protein function. An exception was the HIFs where we anticipated more nuclear staining. Due to the low number of cores with a clear nuclear staining we scored the cytoplasmic expression. In stroma, density was scored as; 1 = low, 2 = intermediate, 3 = high. Throughout scoring procedures, the oncologist entered the scoring

results directly into a excel worksheet and a mean score was calculated for the duplicate cores for each individual. Interindividual variability with respect to IHC-scoring was evaluated on the current material in a previous paper (r = 0.95, range 0.93-0.98).(139) See Table 7 for details regarding expression and scoring.

4.5 Determination of cut-off values

The most frequently used analytical strategy for TMA-studies is subdivision into subgroups based on expression data. Dichotomization into high- and low-groups is easy if expression is only present or not. If, like in our studies, there is a multilevel or even continuous scale, cut-off points are not predefined. Some have used the mean expression and others have tried different cut-off points and then reported on the split giving the most significant difference in outcome between the high- and low-groups. The advantage of the mean is the lower risk of false positive results (Type 1 error) and it might even be easier to reproduce the results by other groups. The disadvantage by using the mean in stead of the most significant cut-off is that the mean cut-off is probably not the biologically important cut-off and the risk of false negative results increases (Type 2 error). A balance of the two important risks of errors is the best. A way to circumvent the drawbacks of the use of the most significant cut-off is to use the *hypothesis-generating* results from a study with the use of the most significant cut-off in a subsequent independent *hypothesis-testing* study.(140) In our studies we have consistently used the most significant cut-offs, while trying to secure large enough subgroups. Therefore, independent validation of our hypothesis generating results would be of great value. Several high-impact journals like *Clinical Cancer Research* explicitly now want retrospective biomarker studies to include a validation study (http://clincancerres.aacrjournals.org/site/misc/journal_ifora.xhtml). We have therefore started to collaborate with another research group with TMAs for another cohort of NSCLC patients.

4.6 Statistical analysis

The dimension of the patient cohort was estimated with disease-specific survival (DSS) as the primary endpoint. The entered criteria (into PASS 2002, Number Cruncher Statistical systems, Kaysville, UTAH) were: 1) At least 50% increase in hazard ratio resulting from the presence of a biomarker was assumed to represent a clinically significant effect; 2) The 5-year DSS for stage I-IIIA NSCLC patients around 60%; 3) The frequency of a biomarker around 35%.

The statistical analyses were done using the versions 15.0, 16.0, 17.0.0 packages from SPSS (Chicago, IL). The $\chi 2$ test and Fishers exact tests were used to examine the associations between molecular marker expressions and the clinicopathological markers. r-values are the Spearman's rank correlation coefficient. Survival curves, according to marker expressions, were drawn using the Kaplan-Meier method, and the statistical significance between survival curves was assessed by the log-rank test. The survival curves were terminated at 120 months in Paper 1 and 146 months in paper 2 and 3, due to less than 10% of patients at risk after this point. The chosen endpoint, DSS, was calculated from time of surgery to time of lung cancer death.

In order to assess the independent value of the tested biomarkers on DSS in presence of established variables, we used the Cox proportional hazard model for multivariate analyses. For all three papers, all significant variables (both biomarker and clinicopathological markers) from the univariate analyses were entered into the multivariate analyses in a backward stepwise Cox regression analysis with a probability for stepwise entry and removal at 0.05 and 0.10, respectively. A $P < 0.05$ was considered statistically significant for all analyses. In subgroup multivariate analyses all variables were again tested for significance in a univariate analysis and only the significant ones in that subgroup were entered in the subgroup multivariate analyses. In Paper 1 a multivariate analysis was done for the whole cohort and

for the two dominant histological subgroups, AC and SCC. For Paper 2 the multivariate analysis was done for the whole cohort (Model 1) and for the subgroup of patients with high Ang-2 expression (Model 2). In Paper 3 the multivariate analysis was done for the whole cohort for both Model 1 and 2, but for Model 2 the biomarkers were entered as a co-expression variable. The co-expression variable, including all of the four HIF hydroxylases, was stratified by the number of HIF hydroxylases demonstrating high expression. All multivariate analyses respected the rule-of-thumb with a minimum of five events (lung cancer deaths) pr entered variable.

5 MAIN RESULTS

5.1 Patient characteristics

Demographic, clinical and histopathological variables are shown in Table 5. The most recent (third) DSS update was done in January 2011, but was not used for the papers in this thesis. The results herein are from the update in November 2008 where 99 patients of the 335 included patients were still alive and 137 patients were dead of lung cancer (DSS event). The median follow-up was 86 months (range 48-216) and the median patient age was 67 years.

Table 5: Patient characteristics and prognostic clinicopathological variables for disease-specific survival as updated in November 2008. P-values represent the log-rank test for significant differences in survival between subgroups.

Characteristic	Patients (n)	Patients (%)	Median survival (months)	5-Year survival (%)	P
Age					0.34
≤ 65 years	156	47	83	55	
> 65 years	179	53	NR	60	
Sex					0.20
Female	82	25	190	63	
Male	253	75	83	56	
Smoking					0.23
Never	15	5	19	43	
Current	215	64	NR	60	
Former	105	31	71	54	
Performance status					**0.013**
PS 0	197	59	NR	63	
PS 1	120	36	64	52	
PS 2	18	5	25	33	
Weight loss					0.71
< 10%	303	90	127	58	
> 10%	32	10	98	57	
Histology					0.08
SCC	191	57	NR	66	
AC	113	34	54	45	
LCC	31	9	98	56	
Differentiation					**< 0.001**
Poor	138	41	47	47	
Moderate	144	43	190	64	
Well	53	16	NR	68	
Surgical procedure					**0.004**
Lobectomy + Wedge*	243	73	190	61	
Pneumonectomy	92	27	37	47	
Pathological stage					**< 0.001**
I	157	47	190	71	
II	136	40	61	51	
IIIa	42	13	17	23	
Tumor status					**< 0.001**
1	85	25	190	74	
2	188	56	84	57	
3	62	19	25	36	
Nodal status					**< 0.001**
0	232	69	190	66	
1	76	23	35	43	
2	27	8	18	18	
Surgical margins					0.29
Free	307	92	190	58	
Not free	28	8	47	47	
Vascular infiltration					**< 0.001**
No	284	85	190	58	
Yes	51	15	27	32	

5.2 Overview of biomarkers and their expression

An overview of biomarkers in our studies and their expression is given in Table 6

Table 6: Expression and scoring of biomarkers

Antigen	Distribution of expression	Localization of expression	Localization scored	Scoring of tumor intensity	Scoring of % positive tumor cells	Scoring of stromal intensity	Scoring of stromal density
HIF1α	Mainly homogenous	Mainly cytoplasmic, minor nuclear (10%) with accentuated cytoplasmic staining	Cytoplasmic	0 = negative 1 = weak 2 = intermediate 3= strong	No	0 = negative 1 = weak 2 = intermediate 3= strong	1=low 2=intermediate 3=high
HIF2α	Mainly homogenous	Mainly cytoplasmic, some nuclear, but at even lower frequency	Cytoplasmic	As HIF1α	No	As HIF1α	As HIF1α
LDH5	Mainly homogenous	Cytoplasmic	Cytoplasmic	As HIF1α	No	As HIF1α	As HIF1α
GLUT1	Heterogeneous and homogenous	Membrane	Membranous	As HIF1α	Yes, but results not reported.	Only expressed in two cores	Only expressed in two cores
CAIX	Very heterogeneous and focal	membrane	Membranous	As HIF1α	Yes, but results not reported.	No stromal cell expression	No stromal cell expression
Ang-1	Homogenous	Mainly cytoplasmic	Cytoplasmic	As HIF1α	No	As HIF1α	As HIF1α
Ang-2	Mainly homogenous	Mainly cytoplasmic	Cytoplasmic	As HIF1α	No	As HIF1α	As HIF1α
Ang-4	Homogenous	Exclusively cytoplasmic	Cytoplasmic	As HIF1α	No	As HIF1α	As HIF1α
Tie-2	Mainly homogenous	Mainly cytoplasmic	Cytoplasmic	As HIF1α	No	As HIF1α	As HIF1α
PHD1	Mainly homogenous	Mainly cytoplasmic, minor nuclear	Cytoplasmic	As HIF1α	No	Not scored due to low-level staining in minority of cells	Not scored due to low-level staining in minority of cells
PHD2	Mainly homogenous	Mainly cytoplasmic, minor nuclear	Cytoplasmic	As HIF1α	No	Not scored due to low-level staining in minority of cells	Not scored due to low-level staining in minority of cells
PHD3	Mainly homogenous, more heterogeneous in BAC	Mainly cytoplasmic, minor nuclear	Cytoplasmic	As HIF1α	No	Not scored due to low-level staining in minority of cells	Not scored due to low-level staining in minority of cells
FIH	Mainly homogenous	Mainly cytoplasmic, minor nuclear	Cytoplasmic	As HIF1α	No	Not scored due to low-level staining in minority of cells	Not scored due to low-level staining in minority of cells

5.3 Paper 1, HIFs and HIF targets

This study aimed to explore the prognostic impacts of the hypoxic markers HIF1α, HIF2α, and the related metabolic markers GLUT1, LDH5 and CAIX in NSCLC as a whole and in histological subgroups. We were able to assess expression profiles of these markers both in tumor and stroma, with the exception of stromal expression for CAIX and GLUT1. Expression profiles are listed in Table 6.

5.3.1 Correlation between markers

There was a strong correlation between the stromal expression of HIF2α and LDH5 ($r = 0.41$, $P < 0.001$). Otherwise there were no relevant correlations ($r \geq 0.2$) between the molecular markers. Between clinicopathological variables and molecular markers, the only highly significant correlation was between high tumor expression of GLUT1 and squamous cell histology ($r = 0.37$, $P < 0.001$). A weak, although significant correlation was observed between increasing T stage and high tumor cell HIF1α expression ($r = 0.15$, $P = 0.005$).

5.3.2 Univariate analyses

Among the examined molecular markers, high tumor cell expression of HIF1α ($P < 0.001$) and GLUT1 ($P = 0.013$) and low stromal cell expression of HIF1α ($P = 0.028$), HIF2α ($P = 0.001$) and LDH5 ($P = 0.11$) correlated significantly with a poor DSS. CAIX expression did not have any significant impact on survival.

For the SCC subgroup, poor differentiation ($P < 0.001$) and increase in pathological stage ($P < 0.001$), T-status ($P = 0.008$) and N-status ($P < 0.001$) were significant clinicopathological prognostic indicators for poor DSS. Among the molecular markers, high

tumor cell expression of HIF1α (P = 0.001) and low stromal cell expression of HIF1α (P = 0.009) and HIF2α (P = 0.005) were significant prognosticators for a poor DSS.

Among AC patients, significant clinicopathological prognostic indicators for a poor DSS were poor WHO performance status (P < 0.001), weight loss (P = 0.025), poor differentiation (P = 0.008), advanced surgical procedure (Pulmenectomy, P = 0.000), increase in pathological stage (P = 0.006), increased N-status (P < 0.001) and vascular infiltration (P = 0.017). Regarding molecular markers, high tumor cell expression of GLUT1 (P = 0.01) and low stromal cell expression of LDH5 (P = 0.03) correlated significantly with a poor DSS. In the subgroup of patients (n = 55) administered postoperative adjuvant radiotherapy, only tumor HIF1α expression tended to be associated with a poor prognosis (P = 0.07).

5.3.3 Multivariate analyses

For all NSCLC patients, higher T-status (P = 0.004), higher N-status (P = 0.001), vascular infiltration (P = 0.03), high tumor cell expression of HIF1α (HR = 2.3, CI 95% 1.3-4.1, P = 0.003) and GLUT1 (HR = 2.0, CI 95% 1.1-3.4, P = 0.02) and low stromal expression of HIF1α (HR = 1.8, CI 95% 1.3-4.1, P = 0.003) and HIF2α (HR = 1.8, CI 95% 1.2-2.8, P = 0.006) were independent significant prognosticators for a poor DSS.

In the SCC subgroup, higher T-status (P = 0.009), poor differentiation (P = 0.001), high tumor cell expression of HIF1α (HR = 3.3, CI 95% = 1.7-6.6, P = 0.001) and low stromal cell expression of HIF1α (HR = 2.1, CI 95% 1.2-3.7, P = 0.008) and HIF2α (HR = 2.3, CI 95%, 1.3-4.4, P = 0.005) were independently and significantly associated with a poor DSS.

In the AC subgroup, higher N-status (P < 0.001), poor WHO performance status (P < 0.001), vascular infiltration (P=0.003), high tumor expression of GLUT1 (HR1.9, CI 95%, P = 0.046) and low stromal expression of LDH5 (HR = 2.3, CI 95%, P = 0.03) were independently associated with a poor DSS.

5.4 Paper 2, Angiopoietins and their receptor

As angiopoietins are being targeted in ongoing clinical cancer trials, we aimed to evaluate the prognostic relevance of all angiopoietins, and their receptor Tie-2 in tumor and stromal compartments of NSCLC tissues from our large unselected cohort of NSCLC patients. Based on the proposed interplay between VEGF-A and angiopoietins, we also examined if the angiopoietins influenced the prognostic impact of VEGF-A expression. Data regarding VEGF-A expression have previously been published by our group.(139) Expression profiles are listed in Table 6.

5.4.1 Correlations

Regarding associations between molecular markers and clinicopathological variables, we found that high tumor cell expression of Ang-4 correlated to histology ($r = 0.19$, $P = 0.003$), as it was more expressed in the squamous histology subgroup. Among examined molecular markers we found tumor cell expression of Ang-4 and Ang-1 to correlate moderately ($r = 0.18$, $P = 0.001$). Further, high tumor cell Ang-2 expression correlated to high tumor cell VEGF-A expression ($r = 0.15$, $P = 0.007$).

5.4.2 Univariate analyses

When assessing associations between molecular markers and disease-specific survival (DSS), we observed high tumor cell expression of Ang-4 ($P = 0.046$) as well as high stromal cell expression of Ang-4 ($P = 0.009$) and Ang-2 ($P = 0.017$) to be associated with a favorable DSS. For tumor cell Ang-2 expression alone there was no influence on survival. The favorable impact of high tumor cell Ang-4 expression was most prominent for subgroups of patients below 65 years ($P = 0.002$), males ($P = 0.027$), squamous cell histology ($P = 0.038$), nodal status 1 ($P = 0.007$) and those without vascular infiltration ($P = 0.015$). For Tie-2 and Ang-1 expression there was no association with DSS.

There was a profound survival impact of high tumor cell VEGF-A expression, but only in patients with concomitantly high Ang-2 tumor cell expression (P<0.001). At low Ang-2 expression, tumor cell expression of VEGF-A had an insignificant prognostic impact (P = 0.078).

5.4.3 Multivariate analyses

In model 1, where all patients were assessed, low stromal Ang-4 (HR = 1.47, CI 95% 1.02–2.11, P = 0.04), low stromal Ang-2 expression (HR = 1.88, CI 95% 1.15–3.08, P = 0.012) and high tumor cell expression of VEGF-A (HR = 1.49, CI 1.04–2.14, P = 0.029) were significant independent prognosticators for poor DSS in addition to several clinicopathological variables (higher T status, P<0.001; higher nodal status, P<0.001; poor performance status, P = 0.013; vascular infiltration; P = 0.011; poor differentiation, P = 0.033). High tumor cell expression of Ang-4 did not reach statistical significance (P = 0.15) with respect to prognosis after adjustment.

In model 2, only patients with high tumor cell expression of Ang-2 were assessed (N = 88). In this subgroup, high tumor cell expression of VEGF-A mediated an independent and strong negative prognostic effect (HR = 6.43, CI 95% 2.46–16.79, P<0.001). As did the following clinicopathological variables: positive nodal status (P = 0.003), reduced performance status (P = 0.024) and poor differentiation (P = 0.034).

5.5 Paper 3, HIF hydroxylases

We aimed to pioneer the first comprehensive prognostic impact evaluation of the HIF hydroxylases in this NSCLC cohort. Studies evaluating the clinical significance of these markers in malignancy are limited, even though they have a potential role as therapeutic targets. Due to available results from our previous NSCLC studies using this cohort, we were

able to examine correlations between the HIF hydroxylases and previously published markers of interest.

5.5.1 Correlations

There were no correlations between HIF hydroxylases and the clinicopathological variables, but weak correlations between some HIF hydroxylases were observed; PHD1 vs.. FIH ($r = 0.14$, $P = 0.013$) and PHD2 vs. FIH ($r = 0.183$, $P = 0.001$). When the HIF hydroxylases were compared with our tumor cell expression data from previously examined angiogenic markers(139;141-144), the following correlations were observed; PHD1 vs. VEGF-A ($r = 0.27$, $P = < 0.001$) and PDGF-A ($r = 0.23$, $P < 0.001$); PHD2 vs. VEGFR3 ($r = 0.23$, $P < 0.001$); FIH vs. VEGFR3 ($r = 0.25$, $P < 0.001$), Notch4 ($r = 0.26$, $P < 0.001$), HIF2α ($r = 0.22$, $P < 0.001$), LDH5 ($r = 0.22$, $P < 0.001$), Ang-1 ($r = 0.21$, $P < 0.001$), Ang-4 ($r = 0.24$, $P < 0.001$), and Tie-2 ($r = 0.22$, $P < 0.001$).

5.5.2 Univariate analyses

We observed high tumor cell expression of all examined HIF hydroxylases (PHD1, $P = 0.023$; PHD2, $P = 0.013$; PHD3, $P = 0.018$; FIH, $P = 0.033$) to be significantly associated with poor survival.

When assessing the co-expression between all the HIF hydroxylases, there was a significant additive pattern with a progressively worse survival by the increasing number (0-4) of highly expressed HIF hydroxylases.

5.5.3 Multivariate analyses

In model 1, we found high tumor cell expression of the PHD2 (HR = 2.03, CI 95% 1.20-3.42, $P = 0.008$) and PHD1 (HR = 1.45, CI 95% 1.01-2.10, $P = 0.047$) to be significant independent poor prognosticators for DSS, in addition to several clinicopathological variables (high T status, $P < 0.001$; high nodal status, $P < 0.001$; poor performance status, $P = 0.001$;

vascular infiltration; P = 0.002; poor differentiation, P = 0.006). High tumor cell expression of PHD3 (P = 0.058) and FIH (P = 0.15) did not, however, reach statistical significance.

In model 2, we found a gradually increasing hazard ratio for lung cancer death in patients with an increasing number of highly expressed HIF hydroxylases in comparison to those without high expression levels. Patients with high tumor cell expression of all four HIF hydroxylases had a HR of 6.48 (CI 2.23-18.8, P = 0.001) compared to patients without high expression of HIF hydroxylases.

5.6 Summary of prognosis data

A summary of the prognostic impact mediated by the investigated markers is presented in Table 7

Table 7: Summary of statistically significant prognosis data from the univariate, multivariate, subgroup and co-expression analyses published in Paper 1-3. Hazard ratios are only stated if significant at the $P \leq 0.05$ level.

Biomarker		**Univariate analyses**		**Multivariate analyses**		**Subgroup or co-expression analyses**
		Tumor	**Stroma**	**Tumor**	**Stroma**	
HIF1α		↓	↑	↓ HR = 2.3	↑ HR = 1.8	Only significant in SCC subgroup, HR tumor = 3.3, HR stroma = 2.1
HIF2α		NS	↑	NE	↑ HR = 1.8	Only significant in SCC subgroup, HR = 2.3
LDH5		NS	↑	NE	NS	Only significant in AC subgroup, HR = 2.3
GLUT1		↓	NSc	↓ HR = 2.0	NE	Only significant in AC subgroup, HR = 1.9
CAIX		NS	NSc	NE	NE	-
Ang-1		NS	NS	NE	NE	-
Ang-2		NS	↑	NE	↑ HR = 1.88	See VEGF-A row for subgroup analyses
Ang-4		↑	↑	NS	↑ HR = 1.47	-
Tie-2		NS	NS	NE	NE	-
PHD1		↓	NSc	↓ HR = 1.45	NE	Patients with high tumor cell expression of all HIF hydroxylases had a very poor prognosis with a HR = 6.48
PHD2		↓	NSc	↓ HR = 2.03	NE	
PHD3		↓	NSc	NS	NE	
FIH		↓	NSc	NS	NE	
VEGF-A *	**High Ang-2**	↓	NA	↓ HR = 6.43	NA	-
	Low Ang-2	NS	NA	NE	NA	-

↑ High expression in compartment associated to good prognosis, ↓ High expression in compartment associated to poor prognosis, NS = Not significant, NSc = Not scorable, NE = Not entered due to non-significance in univariate or not scorable expression in the compartment, NA = Not analyzed, SCC = Squamous cell carcinoma, AC = Adenocarcinoma, HR = Hazard ratio

*Evaluation of expression not primarily part of this study, but assessed in subgroups of Ang-2 expression. Details regarding VEGF-A expression can be found in ref (139)

6 DISCUSSION

6.1 A summary of strengths and weaknesses

The strengths and weaknesses of this study have been discussed in more detail in the material and method sections (section 4). Summarized, strengths and weaknesses are presented in Table 8.

Table 8: Strength and weaknesses of our study

Weaknesses	**Strengths**
Lack of standardized tissue acquisition and fixation protocol	Relatively large number of patients.
Possibly reduced tissue representativity/heterogeneity due to small tissue samples (cores)	Hardly any selection bias due to the fact that near all NSCLC stage I-IIIA patients are surgically treated in the region, and 90% of these were included
No in-house validation of antibody specificity at our lab (mostly commercial antibodies)	A comprehensive collection of vital clinical data
No external positive controls in paper 1 and 2 and only one external positive control for one antibody in paper 3 (PHD1)	The patient follow-up is extensive.
Isotype control was not done for paper 1 and paper 3.	The tissue fixation technique has not been altered during the patient inclusion period
Assessments of tissue staining is semiquantitative	All included tissues were carefully reviewed by two experienced pathologists, bringing the stage classifications up to date
Stroma was only assessed as a whole	We have used few dedicated technicians for TMA assembly and IHC-procedures
Optimal cut-offs were used which increases the risk of false positive results (Type 1 errors)	Duplicate cores taken from preselected and marked areas on the tumor blocks
Possibly most important, our results were not validated in other patient cohorts.	Careful antibody selection and in-house testing was performed
	Expression profiles for the different biomarkers were carefully compared to already published data
	Appropriate positive controls were used where consistent positive tissue was known
	The microscopic scoring for IHC staining was always done by two independent pathologists, blinded to outcome and each other's scores
	Optimal cut-offs reduce false negative results (Type II errors).
	Appropriate and robust statistical analyses were performed, with main outcome being disease-specific survival.
	We assessed protein expressions both in stromal and neoplastic cells

6.2 Paper 1

Herein, we identified the tumor cell expression of HIF1α and GLUT1 to be independent prognosticators for poor survival, whereas stromal expression of HIF1α, HIF2α and LDH5 were prognosticators for a better survival. We observed a discrepancy between histological subgroups as the HIF results were significant in the SCC, but not in the AC subgroup. This observation was contrasted to the LDH5 and GLUT1 results, which were significant in the AC, but not the SCC subgroup.

To explore hypoxia-related markers, HIFs and HIF responsive genes were obvious choices, although there were already several related studies in neoplasias, and also in NSCLC. See Table 9 for a summary of studies regarding these biomarkers and their prognostic relevance in NSCLC. To our knowledge, this is the largest study published in this research field, and the only study examining the prognostic impact of HIF and HIF-related marker expression in stroma. Two of the previous published studies were TMA-based while the others were whole slide studies. Which compartment of the cell to score has in general been fairly easy to determine. However, the HIFs have been scored both in the nucleus and cytoplasm. The nuclear scoring is biologically plausible as HIFs are known to exert their effects in the nucleus. We set out to do both, but less than 10% of cells had nuclear positivity and all cases with nuclear positivity had strong cytoplasmic staining. No other information was found by nuclear scoring than could not be detected by a high cut-off of cytoplasmic staining. In tumor, we chose a high cut-off score for HIF1α positivity. This cut-off score separated the subgroups best with respect to survival, and the high expression group had in general also nuclear staining. Corroborating our findings, high tumor cell expression of HIF1α has in previous studies been associated with a poor survival across a wide range of cancers. In NSCLC, however, none of the previous studies found tumor cell expression of HIF1α to have a prognostic impact independent of clinicopathological or other factors.(59)

This is in contrast to our study. Regarding two studies studying tumor cell expression of HIF1α and HIF2α in NSCLC, both identified HIF2α as an independent prognostic factor.(53;145) No clear explanation for this divergent finding can be provided. The association between high tumor cell expression of HIFs and poor survival has been ascribed to their role as endogenous markers of hypoxia and tumor hypoxia, which is associated to poor survival.(146) However, their role as endogenous markers of hypoxia is disputed.(147)

Hitherto, we are alone in studying the stromal expression of HIFs. We found an inverse prognostic impact between expression of HIFs in tumor and stroma. In stroma, high HIF expression appeared to render a beneficial prognostic impact. An improved prognosis for patients with a high expression of various markers in the stroma has been a consistent finding in our studies.(139;143;144;148-154) We suspect that the *in-situ* stromal immunity may be, at least in part, responsible for these findings. We have previously reported high densities of CD4+ and CD8+ lymphocytes in stroma to be strong independent positive prognosticators in NSCLC, and that lymphocytes constitute a large proportion of NSCLC stromal cells.(153) This may, however, not be the whole story as correlations between lymphocytes and hypoxic markers are only weak to moderate, though present. In addition, some reports have suggested that HIF1α activity in T-cells may protect hypoxic cancerous tissues from anti-tumor T cells.(155) Moreover, HIFs in the stroma had a highly significant prognostic impact in the SCC subgroup, but no prognostic relevance in the AC subgroupgroup. We observed that the SCC subgroup had a marginally higher percentage high expression cases than AC. This can only partly explain some of the difference. On the other hand, SCC tumors are known to be more hypoxic than AC tumors.

Among the three available GLUT1 studies in NSCLC(156-158) only Minami et al.(158) identified GLUT1 as an independent prognostic factor. The larger, but older, study by Younes published only prognostic data regarding the co-expression of GLUT1 and

GLUT3.(156) Extensively expressed in tumor cells, GLUT1 is an important player in cancer metabolism. Due to the high prevalence of high GLUT1 tumor cell expression in SCC, we only detected a statistically trend for reduced survival due to the small number of low expression cases. In ACs we found a significant poor prognostic influence by high tumor cell GLUT1 expression. There was also a slightly larger absolute difference in 5-year survival in the AC when compared to the SCC subgroup. A larger prognostic impact in AC subgroup seems a proper conclusion.

Table 9: Other IHC studies in NSCLC regarding biomarkers in Paper 1. Pubmed accessed 01.08.11

Author	Journal and year	Bio-markers	Patients	Slides	Evaluation of expression	Evaluated compartment and localization	Scoring and cut-off	Univariate analyses	Multivariate analyses
Younes et al.	Cancer 1997	GLUT1	289 (25% SCC, 60% AC) Stage 1	WS	1 observer? Semiquantitative	Membrane, tumor	scale 1-6. All tried, but negative vs >1% positive	GLUT1 and GLUT3 tested together, but probably GLUT1 most important factor (P = 0.0133)	NA
Giatromanolaki et al.	BJC 2001	HIF1α HIF2α	108, T1-2, N0-1 (72 SCC), 36 AC)	WS	2 or 3 observers?, semiquantitative and quantitative	Nuclear and cytoplasmic combined, tumor	Combined score 1-4	High HIF1α (P = 0.08) and HIF2α (P = 0.008) associated with poor overall survival	HIF1α: NS when all variables included. HIF2α significant if HIF1α was excluded
Kim et al.	Lung Cancer 2005 and Clin Cancer Res 2001	HIF1α CAIX	74 (38 SCC, 26 AC, 6 LCC, 4 unspec) Stage I and II, male only	WS	2 observers, semiquantitative and quantitative	HIF1α: Nuclear only, tumor only CAIX: Cytoplasmic?, tumor	HIF1α: % positive cells > mean were positive CAIX: positive if intensity>1, mean cut-off for % cells	HIF1α: High HIF1α associated to poor survival CAIX: High % of positive cells associated to poor survival (P< 0.01)	HIF1α: NS CAIX: As continuous variable, high % of positive cells associated to poor survival (P = 0.005)
Giatromanolaki et al.	BJC 2001	CAIX	107 (70 SCC and 37 AC) T1-2, N0-1	WS	Number of observers not stated, Semiquantitative	Membrane and tumor	% of positive cells	High expression associated to poor survival (P = 0.02)	Significant if highly vascularized cases were excluded (P = 0.02)
Minami et al.	Lung Cancer 2002	GLUT1	47 (AC only), Stage 1	WS	Number of observers not stated	Localization not stated, Tumor	>10% were scored positive	Positive expression associated to poor survival (P < 0.0001)	Positive expression associated to poor survival (P < 0.026)
Lee et al.	J Korean Med Sci 2003	HIF1 α	84 (45 SCC, 39 AC), operable	WS	2 observers, semiquantitative and quantitative	Nuclear, tumor	Sum of intensity (0-3)and %positive (0-3) >2	Non-significant P = 0.442	NA
Giatromanolaki et al.	BJC 2003	LDH5	112 (76 SCC and 36	WS	Number of observers not	Nuclear and cytoplasmic,	Cytoplasmic, median (>80%) was used as	Both high cytoplasmic and high	Only significant as a co-expression

			AC) T1-2, N0-1		stated, Semiquantitative	tumor only	cut-off Nuclear, median (10% used as cut-off)	nuclear expression was associated to poor overall survival (P = 0.03 and 0.02, respectively)	variable with HIF2α
Swinson et al.	Int J Cancer 2004 and J Clin Onc 2003	HIF1α CAIX	172 (107 SCC, 49 AC, 12 LCC), Stage I-IIIA	WS	number of observers not stated observer, Semiquantitative	HIF1α: Nuclear only, tumor only CAIX: perinuclear, membranous, and stromal	HIF1α: % of positive cells. 5% (mean) cut-off, but other tested (>60%) CAIX: pCAIX, pos or neg, mCAIX % of positive cells with quartile cut-offs, sCAIX density of staining 0-4	HIF1α: High positive (>60% positive cells) was associated with poor survival. CAIX: High perinuclear and not membrane CAIX associated with poor survival	HIF1α: NS if standard variables were included CAIX: NS
Hirami et al.	Cancer Letters 2004	HIF1α	80 (58 adeno, 19 SCC)	WS	2 observers, semiquantitative	Cytoplasmic and nuclear, tumor	Combination of intensity and % of positive cells (1-4) ≥3	NS	NS
Nguyen et al.	Eur J Radiol 2007	GLUT1	53 (SCC 19, AC 30)	WS	1 observer, Semiquantitative	Membrane, tumor	combination of % of positive cells and intensity	NS to disease-free survival	NA
Hung et al.	Thorax 2009	HIF1α	87	WS	2 observers, semiquantitative	Nuclear and tumor	>50% positive cells	High expression associated to poor survival (P = 0.005)	Only co-expression with TWIST and Snail was tested.
Kayser et al.	Diagn Pathol 2010	LDH5	269 (106 SCC, 90 AC and 73 LCC)	TMA	1 observer?	Cytoplasmic and/or nuclear tumor	Mean (50%) used as cut-off. Scale 0-3 for intensity and % of positive cells.	NS	NA
Wu et al.	Chin Med J 2011	HIF1α HIF2α	140 (SCC 80, AC 40, 14 other), Stage I-III	TMA	3 observers, Semiquantitative	Cytoplasmic and nuclear, tumor	Combination of cytoplasmic and nuclear (1-4). >2 was high expression	HIF1α: NS HIF2α: High expression associated to poor survival (P = 0.001)	HIF1α: NA HIF2α: High expression associated to poor survival.

NA= Not assessed, WS = Whole slide, TMA = Tissue micro array, SCC = Squamous cell carcinoma, AC = Adenocarcinoma, NS = Not significant. One study regarding GLUT1 was in Chinese only and is not included in this table.(159)

6.3 Paper 2

Herein, we observed the tumor cell expression of Ang-4 to be a prognosticator for poor survival, but not when adjusted for other prognostic variables. In tumor stroma, high expression of Ang-4 and Ang-2 were independently associated with improved survival. When patient subgroups were stratified for high or low Ang-2 expression in tumor cells, we observed high tumor cell expression of VEGF-A strongly correlate to poor survival in the patients with high Ang-2 expression, but not for patients with low Ang-2 expression.

Although Ang-4 expression has not been previously evaluated for prognostic impact, an improved prognosis following high tumor cell expression of Ang-4 is in accordance with an earlier functional *in vitro* study by Olsen and co-workers.(80) They observed Ang-4 to inhibit angiogenesis and reduce the elevated interstitial pressure induced by basic fibroblast growth factor (bFGF) and VEGF in small cell lung cancer tumor cells (GLC19). Recently these results have been contrasted by an observation where Ang-4 promoted glioblastoma progression *in vitro* by enhancing tumor cell viability and angiogenesis.(160)

The prognostic impact of Ang-1, Ang-2 and Tie-2, has been investigated by other researchers. In a fairly large NSCLC study, utilizing IHC to examine the prognostic impact of Ang-1 and Ang-2, Tanaka et al. found that high Ang-2 expression in tumor was associated to poor survival.(134) Due to their heterogeneous staining intensity and low expression in endothelial cells, they were not able, however, to semiquantitatively evaluate the degree of Ang-1 or Ang-2 expression. This is in contrast to the experience by us and others.(131;161) In a smaller IHC study with early stage NSCLC, examining Ang-1 and Ang-2, Reinmuth et al. found high tumor cell expression of Ang-1, but not Ang-2 in tumor to be independently associated with a poor survival.(161) Using IHC and RT-PCR to examine the Ang-2 expression in tumor, Takanami et al. found that high expression of Ang-2 prognosticated poor survival.(162) In other cancers, high expression of Ang-2 in tumor, examined by IHC, has

mostly been associated to a poor prognosis.(130;131;163-165). A balance of Ang-1/Ang-2 expression has been found associated with survival for glioblastoma patients,(166) but not in patients with mammarian cancers.(167)

We also examined the prognostic effects of Ang-2 and VEGF-A co-expressions in tumor cells since VEGF-A has been one of the most studied molecular marker of angiogenesis and the availability of targeted therapies towards VEGF-A. When Ang-2 co-expressed with a high rather than low VEGF-A level, this combination led to a significantly worse 5-year survival (32%) with an HR of 6.43. These results are in accordance with the Tanaka study.(134) Besides, those with a concomitantly low VEGF-A and high Ang-2 expression in tumor cells tended to a better survival compared with those with a low tumor cell Ang-2 expression. These results can be explained by the functional role of Ang-2, as it is known to destabilize the endothelium.(47) The plastic state triggered by Ang-2 can lead to new vessel growth or vessel regression, depending on the presence of factors such as VEGF-A.(72) At low levels of VEGF-A, high Ang-2 levels may lead to vessel regression and a better prognosis. This is in accordance with a study by Huang and colleagues who detected that both over-expression of Ang-1 and administration of an Ang-1 agonist, induced a shift towards Tie-2 stimulation and protected tumors and vasculature from regression.(76) However, in a recent study by Hashizume and co-workers, they observed that inhibition of both Ang-2 and VEGF-A slowed tumor growth more effectively combined than inhibition of any one of them.(168)

Considering our results, it may be speculated if cancers with a high tumor cell Ang-2 expression are more susceptible to anti-VEGF-A treatment (e.g. bevacizumab). The predictive value of angiopoietins and their receptor has only been tested in serum and for effect of chemotherapy in NSCLC with an observation of no predictive value,(169) but for colorectal

cancer patients a low serum level of Ang-2 protein was associated with a better response rate of bevacizumab which is in contrast to our speculations.(170)

In stroma, on the other hand, there are no previous studies examining the prognostic impact of these markers although stromal cells are the main expressors of angiopoietins.(171) In our TMA system systematic studies of tumor vessels could not be done due to the limited stromal tissue of only 0.6 mm in diameter from each patient. Nevertheless, the expression of Ang/Tie-2 markers has been investigated in the stromal compartment where the cross-talk between endothelial cells, fibroblasts, immunological cells and tumor cells are vital for angiogenesis.(47;171) Consistently, we found both Ang-4 and Ang-2 expression in stroma to be independently associated with an improved survival. Since these markers are known to exert opposite effects upon binding to Tie-2, the similar beneficial prognostic effect remains to be elucidated.

Table 10: Other IHC studies in NSCLC regarding biomarkers in Paper 2. Pubmed accessed 01.08.11.

Author	Journal and year	Bio-markers	Patients	Slides	Evaluation of expression	Evaluated compartment and localization	Scoring and cut-off	Univariate analyses	Multivariate analyses
Tanaka et al.	Cancer research 2003	Ang-1, -2	236, Stage I-IIIA	WS	2, observers, %positive cells	Tumor, Heterogeneous expression, cytoplasm	>5% considered positive	High Ang-2 associated to poor survival (p = 0.027). VEGF-A and Ang-2 co-expression associated to poor prognosis (P = 0.004)	High Ang-2 associated to poor survival (HR = 1.46, P = 0.041)
Reinmuth et al.	Lung Cancer2006	Ang-1, 2 and VEGF-A	72, Stage I and II	WS	2 observers, semiquantitative,	Tumor, cytoplasmic	Combined score of intensity and percent, Median cut-off	High Ang-1 associated to poor survival (P = 0.03)	High Ang-1 associated to poor survival (HR = 2.5, P = 0.03) Unadjusted?
Takanami et al.	Oncology Reports 2004	Ang-2	77, curative resection	WS	mRNA and IHC	Tumor cytoplasm	Intensity	High Ang-2 associated to poor survival	High Ang-2 associated to poor survival

6.4 Paper 3

In our final paper, using validated antibodies, we observed scorable expression of all HIF hydroxylases in tumor cells, but not in the stroma. HIF hydroxylases were observed, in general, to be poor prognosticators for NSCLC survival. PHD1 and PHD2 were independent negative prognostic factors in NSCLC. Moreover, there was an additive poor prognostic impact by the increasing number (0-4) of highly expressed HIF hydroxylases.

A limited number studies have evaluated the protein expression of HIF hydroxylases in various cancers(172-180) including one in NSCLC.(181) Only three have assessed survival outcome of cancer in relation to expression of these HIF hydroxylases,(173-175) but none in NSCLC. In general, IHC-studies have found increased, but variable staining of PHDs and FIH in human cancers.(96) Corroborating previous studies, (173;174;176;177;180) we did not find the expected simplistic association between high HIF hydroxylase expression and low HIFα expression, or vice versa, as expected from earlier functional studies.(96). In comparison to the NSCLC study mentioned above,(181) the expression patterns in our TMA cores where similar, but nuclear expression was normally accompanied by a strong cytoplasmic expression. A few small studies in pancreatic cancers(174;175) and prostate cancer, (173) showed associations between HIF hydroxylase expression and reduced survival. A recent gene expression study in NSCLC reported that PHDs were expressed at higher levels in tumor tissue than adjacent normal tissue, but outcomes were not assessed.(182)

The early functional studies on HIF hydroxylases identified them as downregulators of HIFα. The fact that high expression of HIF hydroxylases serve as poor prognosticators for DSS seemingly contradicts the canonical function as inhibitors of HIFα.(59) Several experimental studies have recently tried to elucidate essential roles of HIF hydroxylases in tumor progression. In line with our data, Henze et al. found that inhibition of PHDs significantly reduced glioblastoma cell survival and that PHD inhibition increased hypoxic

cell death as well as death induced by chemotherapeutic agents. Furthermore, the PHD/HIF regulatory axis was postulated as a novel therapeutic target to disable a tumor's ability to adjust to hypoxic conditions and maintain cell survival.(176) Mazzone et al. did functional studies of the stromal role of PHD2 in tumorigenesis by implanting pancreatic tumors in immunocompromized PHD2+/- mice.(183) Surprisingly, the heterozygous deficiency of PHD2 led to improved endothelial lining, vessel maturation, tumor perfusion and oxygenation , and a subsequent inhibition of tumor cell invasion, intravasation and metastasis. The experimentally reduced available level of PHD2 in the host actually seemed to reduce the malignancy of implanted tumors. In accordance, a recent publication by Ameln et al. showed that inhibition of PHD2 in osteosarcoma and melanoma tumor cells actually stimulated vessel formation, but resulted in a profound reduction of tumor growth through the TGFβ pathway.(184) Besides, Ginouves et al. found that chronic hypoxia (24 h to 7 days) increased the pool of PHDs and overactivated all three isoforms thereby "desensitizing" HIFα and protected cells from necrosis.(185) Desensitizing HIFα proved to be required since all experimental cells died if HIF1α expression was not reduced during chronic hypoxia. Chan et al. found that tumors grew dramatically faster than control tumors and that PHD2 loss also induced angiogenesis and recruitment of bone marrow-derived cells in a model of colon carcinoma xeongrafts with decreased PHD2 expression.(186) In pancreatic cancer, Su et al. reported that PHD3 overexpression mediated tumor cell growth and invasion,(179) whereas overexpression of PHD1 was shown by Erez et al. to inhibit tumor growth.(187) The studies so far do not give us a clear functional explanation of the HIF hydroxylases in cancer. To quote Jokilehto and Jaakkola in a recent review "given the uncertainties in specific PHD function, their role in cancer is inconclusive at the best".(96)

As high cellular levels of HIF hydroxylases seem to be important in the malignant phenotype, they may qualify as potential therapeutic targets in NSCLC. Due to the basic

understanding of HIF hydroxylase functions, Nagel et al. recently proposed inhibition of HIFα through activation of PHDs.(188) But if PHDs hold a position in disease progression, consistent with our findings, it would rather be a target of inhibition. Although several inhibitors of HIF hydroxylases are known, there are presently no registered ongoing studies on such inhibitors in cancer registered on (www.clinicaltrials.gov accessed 05.09.11).

7 CONCLUSIONS AND IMPLICATIONS FOR FURTHER RESEARCH

Hypoxia is a common phenotype in NSCLC and leads to adaptive cell changes in surviving cancer cells. Angiogenesis is one of the most important adaptive phenotypes employed by hypoxic tumors involving central angiogenic markers like angiopoietins (Angs), vascular endothelial growth factors (VEGFs) and their receptors.

Hypoxia is associated to chemo- and radio-resistance and ways to reverse or exploit hypoxia in cancer have been a focus for cancer research for more than five decades. Hypoxia in actual patient tumors has been difficult to investigate in routine clinical practice, so efforts have been made to identify markers of hypoxia. Hence, the term endogenous markers of hypoxia, has been used for hypoxia related biomarkers. Hypoxia induced factors (HIFs), their subsequent target genes and angiogenic markers are at the heart of adaptive cancer cell mechanisms. Their prognostic or predictive roles will hopefully improve the understanding of tumor biology, improve patient prognostication and facilitate personalized and more optimal therapy as well as assisting future drug developments and drug trials.

In this thesis we have studied the hypoxia inducible factors (HIFs), several of their target genes (CAIX, LDH5, GLUT1 and HIF hydroxylases) and important players in angiogenesis (angiopoietins and their receptors). Among these we have identified important independent prognostic factors in tumor and stromal tissue as well as finding these results to be associated to important histological subgroups. The co-expression of oxygen sensors in NSCLC tumors was observed to be a strong independent variable to effectively separate patients with regard to prognosis.

Our study is not perfect and our weakest spots are the lack of validation cohorts and sparse in-house antibody validation. Hitherto, our NSCLC cohort is, however, one of the largest contributing in the quest for useful biomarkers. To our knowledge, it is the only

prognostic study in NSCLC evaluating prognosis through stromal marker expression. Finally, a lot of effort has been put into quality assuring measures.

The findings herein will hopefully be tested in prospective studies. We also hope that the evaluated biomarkers will gain attention with respect to future clinical trials targeting these molecules in cancer so that also the predictive role of these biomarkers can be elucidated. We are increasingly collaborating with other research groups with TMAs in NSCLC so that validation of each others biomarker results can be achieved. Our group is also prospectively collecting fresh NSCLC tumor tissues for future studies, are establishing short term primary cell lines for molecular characterization and interventions, and we have recently started exploring the emerging field of microRNAs in NSCLC.

We are also broadening our research field by implementing our established methods into new tumor origins like prostate cancer. The years ahead will be demanding in work, but rewarding in new questions and answers.

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