Hypoxia in prostate cancer:

Gene expression profiling in relation to disease aggressiveness and treatment interventions

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Table of contents

1. Preface

1.1 Abbreviations

ADT	androgen deprivation therapy
AR	androgen receptor
ARCON	accelerated radiotherapy with carbogen and nicotinamide
ARE	androgen responsive element
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and rad3 related protein
CCN	cyclin
CDK	cyclin dependent kinase
CT	computerized tomography
DHT	dihydrotestosterone
DNA-PKcs	DNA-dependent protein kinase, catalytic subunit
DSB	double strand break
EGFR	epithelial growth factor receptor
EMT	epithelial to mesenchymal transition
GEO	gene expression omnibus
G_n-phase	gap phase
GnRH	gonadotropin-releasing hormone
GO	gene ontology
HAT	histone acetyltransferase
HDAC	histone deacetylase
HG-PIN	high-grade prostatic intraepithelial neoplasia
HIF1	hypoxia inducible factor 1
HIF1α	HIF1, alpha subunit
HR	homologous recombination
HRE	hypoxia responsive element
HSP	heat shock protein
IMRT	intensity modified external beam radiotherapy
LET	linear energy transfer
LIMMA	Linear Models for Microarray data
MIN	microsatellite instability
M-phase	mitosis (phase)
MRI	magnetic resonance imaging
mTOR	mammalian target of rapamycin
NHEJ	non-homologous end joining
OER	oxygen enhancement ratio
ORP	open retropubic prostatectomy
PHD	prolyl hydroxylase
pO_2	oxygen partial pressure
PSA	prostate specific antigen

qRT PCR	quantitative real time polymerase chain reaction
RALP	robot-assisted laparoscopic prostatectomy
RB1	retinoblastoma protein
ROS	reactive oxygen species
SAM-GS	significance analysis of microarrays for gene sets
S-phase	synthesis phase
SSB	single strand break
TNM	tumor, node, metastasis
TP53	tumor protein p53
TUR-P	transurethral resection of the prostate
VHL	von Hippel-Lindau protein
γH2AX	phospho histone H2AX

1.2 List of papers

Papers included in the thesis:

Paper I: **Ragnum HB**, Vlatkovic L, Lie AK, Axcrona K, Julin CH, Frikstad KAM, Hole KH, Seierstad T, Lyng H. The hypoxia marker pimonidazole reflects a transcriptional program associated with aggressive prostate cancer. *Manuscript.*

Paper II: **Ragnum HB**, Røe K, Holm R, Vlatkovic L, Nesland JM, Aarnes EK, Ree AH, Flatmark K, Seierstad T, Lilleby W, Lyng H. Hypoxia-independent downregulation of hypoxia-inducible factor 1 targets by androgen deprivation therapy in prostate cancer. Int J Radiat Oncol Biol Phys. 2013; 87: 753-60.

Paper III: **Ragnum HB**, Jonsson M, Julin CH, Frikstad KAM, Clancy T, Wennerstrøm AB, Stokke T, Røe K, Ree AH, Flatmark K, Lyng H. Vorinostat-mediated radiosensitization and transcriptional effects in hypoxia-treated prostate cancer cell lines. *Manuscript.*

Other publications by the candidate not included in the thesis:

Røe K, Bratland Å, Vlatkovic L, **Ragnum HB**, Saelen MG, Olsen DR, Marignol L, Ree AH. Hypoxic tumor kinase signaling mediated by STAT5A in development of castration-resistant prostate cancer. PLOS One. 2013; 8: e63723.

Gibbs C, **Ragnum HB**, Svindland O. A man in his 50's with hypotension and hyponatremia. Tidsskr Nor Laegeforen 2011; 131: 2009-11.

1.3 Aim of the thesis

Adenocarcinoma of the prostate, in this thesis referred to as prostate cancer, is the sixth leading cause of cancer-related deaths in males world-wide and the second-leading in Norway (1, 2). After introduction of the prostate-specific antigen (PSA) test, there has been a marked increase in prostate cancer incidence, carrying a risk of over-treating indolent cancers (3). Thus, both selection of correct treatment intensity and improved strategies to overcome treatment resistance are warranted. Hypoxia is associated with poor clinical outcome after curatively intended treatment of prostate cancer, and is especially known to induce tumor resistance against radiotherapy (4, 5). It is as such both a potential biomarker for disease outcome, and a possible target for new treatment combinations and strategies.

Importantly, to fully exploit hypoxia as biomarker for disease aggressiveness, and to optimize treatment strategies against hypoxic tumors, a better understanding of the molecular background of hypoxic prostate cancers is required. Alteration of the transcriptional program is an important part of the cellular stress response to hypoxia, partly mediated by activation of the hypoxia inducible factor 1 (HIF1) transcription factor (6). Evaluation of gene expression can therefore reveal mechanisms by which cells adapt to hypoxia, and give molecular information for increased propensity to invade and metastasize. Thus, the overall aim of this thesis was to investigate whole-genome transcriptional programs in prostate cancer related to hypoxia and treatment interventions. More specifically, we aimed to:

- Contribute to establishment and implementation of a clinical protocol optimized to investigate associations between hypoxia and aggressiveness in prostate cancer patients, and analyze the molecular background of hypoxic tumors with focus on transcriptional changes.
- Investigate transcriptional effects by the treatment interventions androgen deprivation therapy and the histone deacetylase inhibitor vorinostat, with focus on HIF1 signaling.
- Evaluate radiosensitization by vorinostat in hypoxic prostate cancer cell lines.

2. Background

2.1 Hallmarks of cancer

Carcinogenesis, the creation of cancer, occurs due to mutations in DNA which are accumulated over time and passed on to cell progeny (7, 8). These mutations can take form as amplifications, deletions, translocations and nucleotide changes. Genes that promote carcinogenesis when the activity of their gene product is increased are called oncogenes and proto-oncogenes prior to the altering mutation, whereas tumor suppressor genes stimulate carcinogenesis when their activity is reduced or lost. In addition, a third class of genes involved is stability genes, which normal function is to keep mutations at a minimum (9).

The process where a malignant tumor develops is characterized by clonal evolution, where cells with alterations conferring a growth advantage will dominate the tumor and eventually outgrow the other cells (10). These heritable changes result in certain characteristics of the cancer cells often referred to as the hallmarks of cancer, a concept introduced by Hanahan and Weinberg in 2000 (11) and further developed in 2011 (Figure 1; (12))

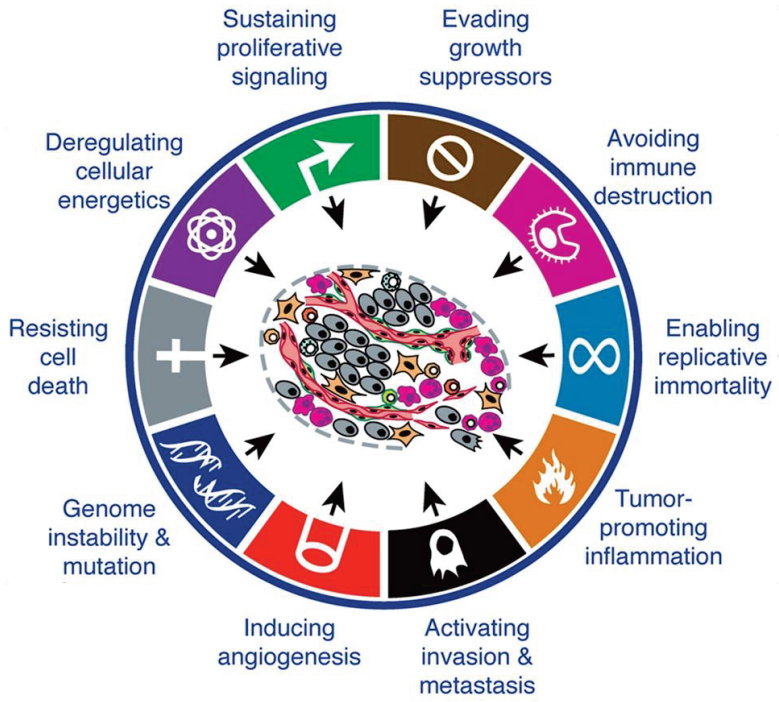

Figure 1: The hallmarks of cancer. Adapted from (12), with permission.

It is assumed that the acquisition of cancer hallmarks is made possible by two enabling characteristics: Genome instability and mutation and tumor promoting inflammation (12). The former hallmark can be acquired through disruption of DNA damage sensors or repair genes by deleterious mutations, and it has the potential of accelerating the rate of favorable mutations (12). One example is mutation of the tumor suppressor protein TP53, often referred to as the "guardian of the genome", which normally induces growth arrest or cell death after irreversible DNA damage (13). As a result of *TP53* mutation, cancer cells may replicate damaged DNA and pass the damage on to progeny, which may lead to further genomic instability and mutations in the daughter cells. Interestingly, in prostate cancer, *TP53* mutations are more common in advanced stages and it correlates with higher grade and poor survival following therapy (14). It is also acknowledged that epigenetic regulation, i.e. heritable changes in gene activity not accompanied by changes in DNA sequence, may give rise to genomic instability, for instance by epigenetic silencing of the cell cycle regulator protein CDKN2A (p16) (15).

Hanahan and Weinberg state that tumor promoting inflammation may be an enabling characteristic of cancer that drive the carcinogenic process by secretion of mutagenic chemicals and supplying bioactive molecules in the microenvironment (12). Conversely, tumor cells must evade destruction by the host immune system, which for instance can be performed through active secretion of immunosuppressive factors (12).

Genome instability and mutations may also be a consequence of tumor hypoxia. This, together with deregulating cellular energetics, inducing angiogenesis, resisting cell death and activating invasion and metastasis are hallmarks of particular relevance for hypoxia-related aggressiveness, and will be further described in the hypoxia section. Moreover, enabling replicative immortality, sustaining proliferative signaling, and evading growth suppressors are hallmarks related to rapid cell proliferation, which is another characteristic of prostate cancer aggressiveness (16, 17). Control of the cell cycle is central in regulating cell proliferation and in maintenance of tissue homeostasis, and this will be covered in the following section.

2.1.1 The cell cycle and tumor cell proliferation

Before mitosis where the cell divides, it must replicate its DNA. The cell cycle describes the order of these events and consists of two phases, interphase and mitosis (M-phase), where interphase is further sub-divided into the G_1-, S- and G_2-phases (Figure 2; (18)).

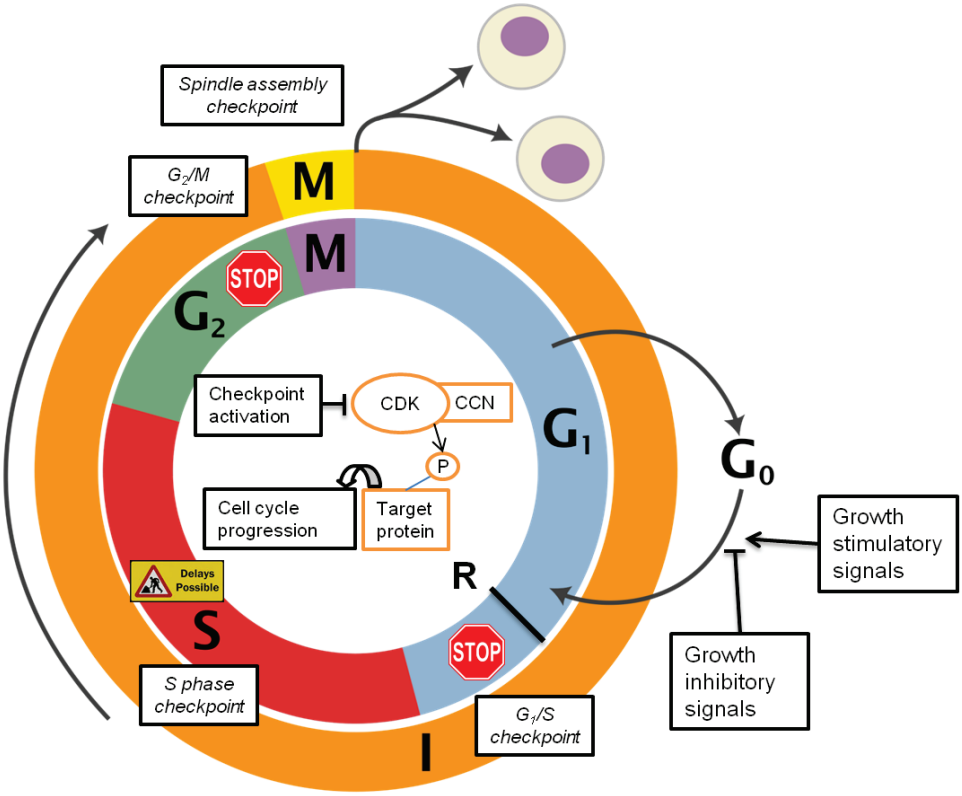

Figure 2: Simplified overview of the cell cycle including checkpoints and regulatory proteins. Adapted from Wikipedia. Abbreviations: G_n: Gap phases, R: Restriction point, S: Synthesis phase, M: Mitosis, I: Interphase, CDK: Cyclin-dependent kinase, CCN: Cyclin, P: Phosphate group.

In S-phase (synthesis phase), DNA is replicated to produce two identical sister chromatids per chromosome. The sister chromatids are split by the spindle apparatus during M-phase. Between these two phases are the gap phases; G_1 between M- and S-phase and G_2 between S- and M-phase. Progression through the cell cycle is strictly regulated through the activity of proteins termed cyclins (CCNs) and cyclin-dependent kinases (CDKs). As the name implies, the expression of cyclins will change during the cell cycle, and each cyclin has a role in a specific phase of the cycle (Figure 2). After hetero-dimerization of a cyclin to a CDK, the CDK phosphorylates serine and threonine residues on target proteins, thus changing their function to allow cell cycle progression. This activity can be further enhanced or attenuated by phosphorylation or dephosphorylation of the CDKs by regulatory enzymes (19).

To counteract the potentially deleterious consequences of replication or chromosome segregation with damaged DNA, cells have a checkpoint apparatus located at G_1/S and G_2/M transitions (20). In addition, there is a spindle assembly checkpoint in M-phase ensuring that anaphase does not occur prematurely (21), and mechanisms for a delay of S-phase progression (22) (Figure 2).

Cell cycle control is vital to tissue integrity, including the decision to enter interphase from a non-proliferative, quiescent state (G_0). The decision to enter the cell cycle from G_0 is the result of a balance between stimulating and inhibiting factors. A balance in favor of stimulating factors (mitogens) will result in upregulation of cyclin D, which dimerizes with CDKs 4 and 6 (Figure 2). This leads to phosphorylation of the retinoblastoma protein (RB1) and release of the transcription factor E2F. E2F stimulates the transcription of cyclin E (CCNE), which acts in a positive feedback loop on RB1, leading to augmentation of free E2F. The cell has now passed the so-called restriction point in G_1 (Figure 2), where it is bound to enter S-phase despite disappearance of mitogenic signaling (18).

Control over the restriction point is related to the cancer hallmarks sustaining proliferative signaling and evading growth suppressants. Proliferative signaling may be conveyed through upregulation of receptors that receive proliferation signals from surrounding tissues, making them sensitive to low levels of ligands, or by mutations that reduce the ligand specificity. Alternatively, the cells may have mutations in signaling cascades downstream of the receptors that render them "always on" (12), or they may produce the ligand themselves (23). In prostate cancer such changes are commonly found in the androgen receptor (AR) or its downstream proteins (24). With regard to the restriction point, AR has been found to upregulate cyclin D (CCND) through the mammalian target of rapamycin (mTOR) pathway, thus stimulating G_1/S transition (25).

Conversely, proliferation, or passing of the restriction point, can be hindered by growth suppressants, and evasion of growth suppressive signaling is one of the cancer hallmarks. Non-transformed cells are often sensitive to growth inhibitory signaling (Figure 2) by for instance transforming growth factor, beta 1 (TGFβ1). Loss of sensitivity to such inhibitory signaling is associated with the carcinogenic process (12, 26).

In culture, non-malignant cells can only divide a limited number of times (27), whereas cancer cells have the ability to divide indefinitely (12). This replicative immortality characteristic is attributed to the function of telomerases, which are enzymes that extend the telomers, i.e. the ends of the DNA molecule. Telomerases counteract the shortening of the

DNA strands that normally occurs at each cell division and leads to cell senescence, i.e. stop in proliferation capacity or clonogenic death (12).

The DNA damage response in relation to radiobiology

The DNA damage response consists of a network of interacting pathways committed to sense DNA damage and elicit counteracting effects (Figure 3, (28)). Sources of DNA damage can be endogenous due to replication stress or production of reactive oxygen species (ROS) in metabolic processes, or exogenous by ionizing radiation, ultraviolet light or chemotherapy (Figure 3). DNA damage which progresses to progeny cells can give rise to cancer development or cell death, and the DNA damage response is as such a tightly regulated system (29).

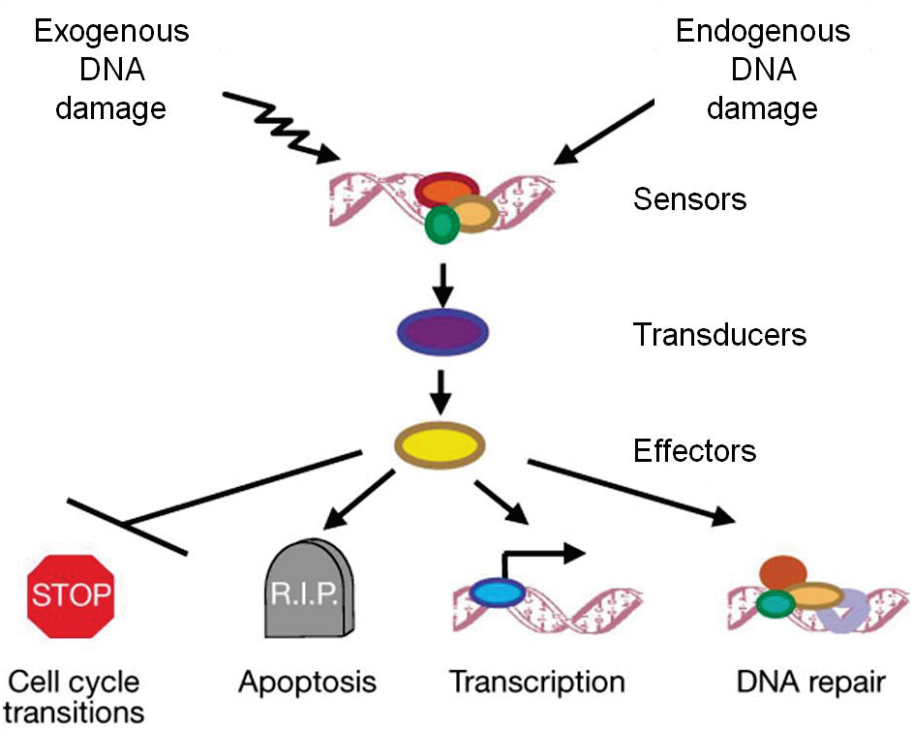

Figure 3: The DNA damage response divided into sensors, transducers and effectors with different effects listed. Adapted from (28), with permission.

Radiotherapy, where ionizing radiation is utilized to induce DNA damage, will elicit the DNA damage response. A radiation dose of 1 Gy causes approximately 20-40 double strand breaks (DSBs) (30). DSBs, which are the most lethal forms of DNA damage, can lead to disastrous changes in the DNA molecule, such as loss of an entire chromosome arm. In DSB repair, one of the first events is the phosphorylation of hundreds of proteins, with one of the earliest being histone H2AX, forming gamma H2AX (γH2AX). Three related kinases are known to be capable of phosphorylating H2AX, namely ataxia telangiectasia mutated (ATM), DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) and ataxia telangiectasia and Rad3-related protein (ATR) (30). ATM is dependent on the DNA damage sensor MRN, consisting of the nuclease MRE11, the DNA-binding protein RAD50 and the ATM-interacting NBS1 in the process of creating γH2AX around DSB foci (30).

Subsequently, ATM may phosphorylate the TP53-MDM2 protein complex. This leads to dissociation and transactivation activity of TP53, which in turn may induce G_1 arrest or in some rapidly proliferating tissues apoptosis (30). ATM and ATR may also phosphorylate the checkpoint kinases CHK1 and CHK2. This may lead to cell cycle delay in S-phase and arrest in G2 phase due to inactivation of CCN/CDK complexes through phosphorylation of CDC25 (30), allowing time for DNA repair. Due to the frequent mutated status of *TP53*, and thereby impaired capability of G_1 arrest, following ionizing radiation cancer cells largely depend on the G_2/M checkpoint in order to repair DNA damage prior to mitosis (31).

The two main pathways for repair of DSBs are homologous recombination (HR) and non-homologous end joining (NHEJ), where HR is only achievable when sister chromatids are available in S- and G_2-phase (Figure 4, (30)). Both HR and NHEJ utilize several proteins in the repair process (Figure 4). HR uses an undamaged sister chromatid as template for repair, and is as such error-free, whereas NHEJ will lead to ligation of the DNA ends after end-processing, making room for mutations. Choice of which pathway to use in the S- and G_2-phases is thought to be determined by the competitive binding of the MRN complex and the KU proteins (XRCC5 and XRCC6) to the DSB site (Figure 4). The KU proteins are important DSB sensors which recruit the DNA-PKcs (30). If DNA repair is not successful, cells can execute the apoptotic process, i.e. undergo programmed cell death.

Other DNA damage forms induced by ionizing radiation include single strand breaks (SSBs), approximately 1000 breaks per Gy, and damage to DNA bases, with more than 1000 in number per Gy (30). If a SSB is situated close to a DNA base damage, the SSB may be converted to DSB in the repair process of the base damage. In addition, induction of SSBs

during DNA replication can lead to collapse of the replication fork and the creation of a single-ended DSB requiring HR (30).

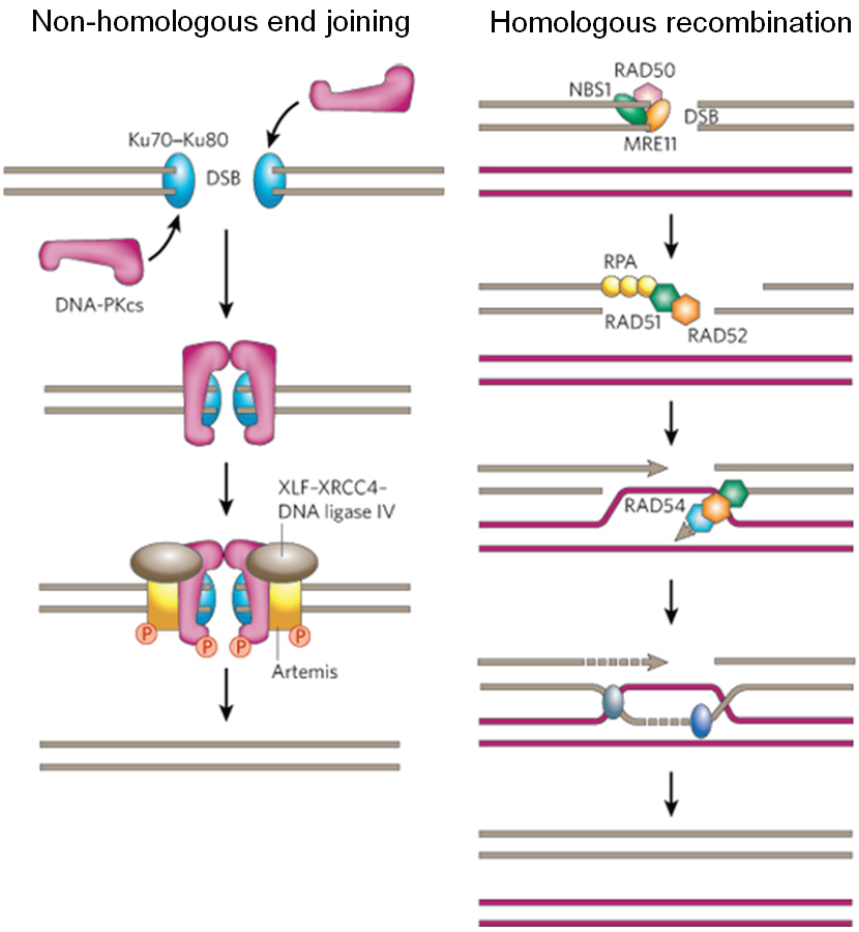

Figure 4: Simplified overview of the two main pathways of DNA double strand break (DSB) repair: Non-homologous end joining (NHEJ) and homologous recombination (HR). In the process of NHEJ the DSB is recognized by the KU proteins and DNA-PKcs. This leads to phosphorylation (P) and recruitment of repair proteins with subsequent ligation of the DNA strand (left). In HR the DSB is recognized by the MRN complex (NBS1, RAD50 and MRE11). The DNA ends are resected and following recruitment of DNA repair proteins, a homologous region on a sister chromatid is used as template resulting in error-free repair (right). Image and legend adapted from (32), with permission.

In radiotherapy, the goal is to target the tumor with ionizing radiation while sparing surrounding normal tissue. This can in part be achieved through conforming of the radiation dose to the tumor. In addition, radiotherapy with curative intent is often given in 2 Gy daily fractions, which counteracts long-term side effects from normal tissues, e.g. the rectal wall, and allows delivery of a greater total dose to the irradiated field (30). Other strategies to increase disease control include concurrent treatment with chemotherapy, which may contribute in several ways, among them through spatial cooperation, where chemotherapy removes tumor cells in other compartments than covered by radiation. Enhancement of tumor response is another mechanism, due to inhibited DNA repair and shrinkage of the tumor (33). In recent years, radiotherapy combined with targeted therapeutics such as antagonists to the epidermal growth factor receptor (EGFR) have gained increasing attention (34).

In general, in addition to tumor size, there are three main phenotypes determining the radiosensitivity of a tumor: Intrinsic radiosensitivity, repopulation between fractions and tumor hypoxia (30). The intrinsic radiosensitivity shows great variation between different cancers and is dependent on alterations in cell cycle checkpoint signaling, apoptosis mechanisms and DNA repair pathways. Repopulation means the ability a tumor has to proliferate between treatment fractions. A high repopulation rate is regarded as a negative prognostic factor (30), and a possible strategy to counteract this is by accelerating the radiotherapy regime, i.e. give the patient more than five fractions of two Gy per week (30). Tumor hypoxia plays fundamental roles in cancer biology in general, including response to ionizing radiation, and is covered in the following section.

2.1.2 Tumor hypoxia

In 1955, after investigation of the histological architecture of lung tumors, Thomlinson and Gray proposed that hypoxia may occur in tumors *in vivo* (35). Since then it has become evident that hypoxia is a common feature of most solid tumors, often associated with increased cancer aggressiveness (36). Hypoxia in cancer biology is often defined as an interstitial oxygen partial pressure (pO_2) below 2 %, compared to a pO_2 of 2-9 % in most normal tissues (37). Other oxygen levels are, however, also used, and the hypoxia term is not well defined (38). Related characteristics of the microenvironment are nutrient deprivation, low extracellular pH and high interstitial fluid pressure (39).

Development of the hypoxic phenotype

Tumor hypoxia is caused by an imbalance between oxygen demand and supply. The oxygen demand of the tumor cells is increased due to their high proliferation activity, whereas a poor vascular network leads to an insufficient oxygen supply. Angiogenesis, the process where new blood vessels form from pre-existing vessels, is normally a balanced process consisting of favoring and opposing signaling, controlled by angiogenic proteins like VEGFA and angiostatin, respectively (40). To maintain oxygen delivery, a growing tumor must induce the generation of its own vascular network. Acquisition of this ability is often referred to as the angiogenic switch (41). Thus, in the development of a tumor, stimulation of existing capillaries to divide and infiltrate a growing neoplasm is a crucial step, and inducing angiogenesis is one of the cancer hallmarks as defined by Hanahan and Weinberg (12). However, the tumor-induced blood vessel network is often highly irregular with tortuous vessels, blind ends and presence of arterio-venous shunts (42). This can lead to sluggish blood flow and episodes of thrombosis causing abrupt changes in blood flow (Figure 5) giving rise to acute, or perfusion-limited, hypoxia (43). Causes of acute hypoxia can dissolve, and thereafter re-emerge – creating cycles of acute hypoxia episodes, often referred to as cyclic hypoxia. Oxygen has a limited diffusion capacity in a tissue, estimated to approximately 145 µm (37) and hypoxia developed by limited diffusion of oxygen due to long distance between functional vessels and cells, is referred to as diffusion-limited, or chronic, hypoxia.

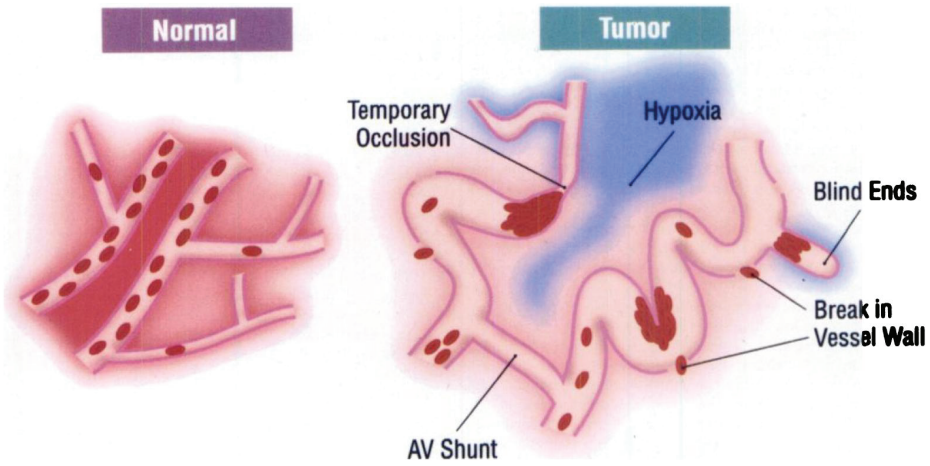

Figure 5: Vasculature in normal tissue and in a hypoxic tumor. Abbreviation: AV Shunt: arterio-venous shunt. From (42), with permission.

For the cells to survive at low oxygen levels, adaptation mechanisms have to be activated. Thus, the hypoxic microenvironment exerts a selection pressure on the tumor cells, resulting in survival of cells with advantageous traits. For instance, it has been shown that cyclic hypoxia will select cells with insensitivity to mitochondria-mediated apoptosis (44). Additionally, Graeber and co-workers demonstrated that hypoxia enriches for cells with mutations in *TP53* (45). This may be an underlying mechanism for the cancer hallmark resisting cell death (12).

Another important survival mechanism under hypoxia is the ability to reprogram metabolism to allow production of ATP despite limited supply of oxygen. This can be achieved through conversion of glucose to lactate. One of the mechanisms involved is stabilization of the alpha subunit of the transcription factor HIF1 (HIF1α). In turn, this leads to increased transcription of glycolytic enzymes, increased glycolytic flux, and thereby sustained ATP production under hypoxic conditions (46).

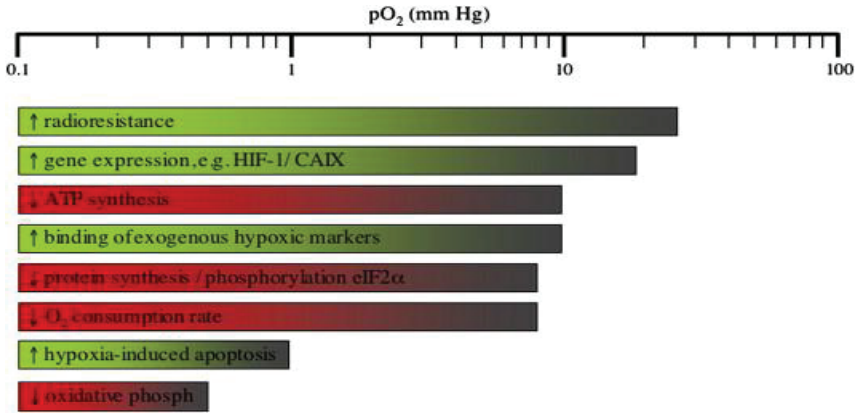

Figure 6: Cellular consequences of hypoxia, with relevant oxygen partial pressures (pO_2) in mmHg on a logarithmic scale, where 7.5 mmHg ≈ 1kPa ≈ 1% O_2. From (38), with permission.

The observation of increased glycolytic flux in cancer is not new, as Otto Warburg described this already in 1924, relating it to mitochondrial dysfunction (47). In later years it has become clear that a reprogramming of metabolism can occur in cancer cells irrespective of hypoxia, thus, deranged energetics is now regarded as one of the cancer hallmarks (12). In terms of ATP production, converting glucose to lactate is less efficient than forming pyruvate with subsequent oxidative phosphorylation (12). However, potential benefits of such a

reprogramming may be a high availability of metabolism intermediates that can be converted to amino acids, nucleotides and lipids needed for proliferation (48) and increased resistance against rapid fluctuations in the oxygen level that would otherwise impair energy production and lead to production of ROS (49). Interestingly, AR, which is utmost important for proliferation and survival of prostate cancer cells, has a stimulatory effect on regulatory, glycolytic enzymes (50).

Hypoxia and radioresistance

Already in 1909, Gottwald Schwarz observed that inducing anemia would make a tissue more radioresistant (51), and in 1953 had Gray and colleagues established that radiation of anoxic tissues caused less damage than radiation under well oxygenated conditions (52). The causal relationship between tumor hypoxia and reduced cell kill by radiation is well established, and tumor hypoxia is an adverse factor in radiotherapy of several cancer forms (53-57), including prostate cancer (4, 5).

The radiation dose needed to reduce the clonogenic capacity to a certain level under hypoxic as compared to normoxic conditions is termed the oxygen enhancement ratio (OER) and is typically reported to be between 2.7 and 3.0 (58). This means that approximately 3 times higher radiation dose is needed to kill the same fraction of cells in a hypoxic than a normoxic milieu. However, OER will vary based both on the linear energy transfer (LET) of the radiation (30), i.e. the energy the radiation particles transfer to the environment per distance, and on the oxygen level. Whereas hypoxia important for cancer biology is often regarded as pO_2 below 2 %, the radiobiologically relevant hypoxia comes into play at lower concentrations, typically below 0.7 % (58).

The mechanism underlying the higher radiosensitivity of cells in the presence of oxygen is explained through the oxygen fixation hypothesis, proposed in the 1950s (30). Radiation causes either a direct damage to the DNA strand, or damages DNA indirectly through ionization of other molecules, mostly water, which again ionizes the DNA strand. Following this, oxygen fixes the DNA damage by reacting with free radicals in DNA, making the damage irreparable by the DNA repair machinery (30). Moreover, the induced damage could be more extensive under oxic conditions, due to increased generation of radicals from free oxygen in addition to radicals from the water molecule.

Several attempts have been made to counteract the reduced efficiency of radiation in hypoxic tumors (58). One way is to diminish chronic hypoxia by letting the patient breathe oxygen enriched gases like carbogen (mixture of 95 % oxygen and 5 % CO_2) or hyperbaric

oxygen to increase the blood oxygen level (59, 60). In the accelerated radiotherapy with carbogen and nicotinamide (ARCON) study on head and neck cancer, nicotinamide was also added, since this drug has been demonstrated to reduce the opening and closing of blood vessels and hereby cyclic hypoxia due to fluctuations in blood flow (61, 62). In a phase three trial, an increased regional control rate was seen for patients on ARCON treatment compared with the control arm with accelerated radiotherapy alone. In a sub-analysis, hypoxic tumors showed a benefit from ARCON, whereas non-hypoxic did not (63). Another strategy for combating hypoxia-induced radioresistance is the use of chemical modifiers that mimic oxygen. This field has evolved since the first experiments by Adams in 1963 (60, 64). Today, the drug nimorazole is included in radiotherapy regimens for head and neck cancer in some hospitals, among them our institution, and has been shown to increase both the local control rate and the disease-specific survival following radiotherapy of patients with hypoxic head and neck tumors (65).

Hypoxia and genetic instability

Hypoxia is thought to affect genetic stability in several ways and has been linked to genome instability and mutation, one of the enabling hallmarks of cancer (12, 66, 67). As mentioned can hypoxia increase the prevalence of *TP53*-mutated cancer cells (45), resulting in deregulated genomic control through reduced cell cycle checkpoint and apoptotic abilities.

Studies have also shown increased rate of spontaneous DNA mutations in cells exposed to hypoxia, which could be due to induction of intrachromosomal fragile sites (68). Furthermore, cyclic hypoxia, where episodes of acute hypoxia are followed by reoxygenation and massive outburst of free radicals, may damage DNA. DNA repair enzymes are found to be downregulated under hypoxia, especially enzymes important for HR, such as RAD51, resulting in increased chromosomal instability (66). Hypoxia has also been associated with another mechanisms for DNA instability, namely microsatellite instability (MIN) (66). MIN is caused by defective mismatch repair, one of the DNA repair pathways, which is important for repair of base substitutions and misalignments occurring during DNA replication (66).

Hypoxia, invasion and metastasis

The main cause of death from cancer is by metastasis to other organs (69), and there is a growing body of pre-clinical evidence and clinical associations linking hypoxia to higher invasive potential and ability to metastasize (36). The underlying mechanisms have not been resolved, and seem to differ across tumors. However, both hypoxia induced genomic

instability and activation of signaling pathways through regulation of gene and protein levels and activity may play a role (67). Activating invasion and metastasis is one of the cancer hallmarks (12), and for carcinoma cells it starts by invasion through the basement membrane into the surrounding stroma. Subsequently, cancer cells must acquire mutations and epigenetic alterations which allow them to intravasate, survive in the blood or lymph circulation, arrest at a distant organ site and extravasate into a new tissue (Figure 7, (69)). At these steps, hypoxia has shown to be a key player (70).

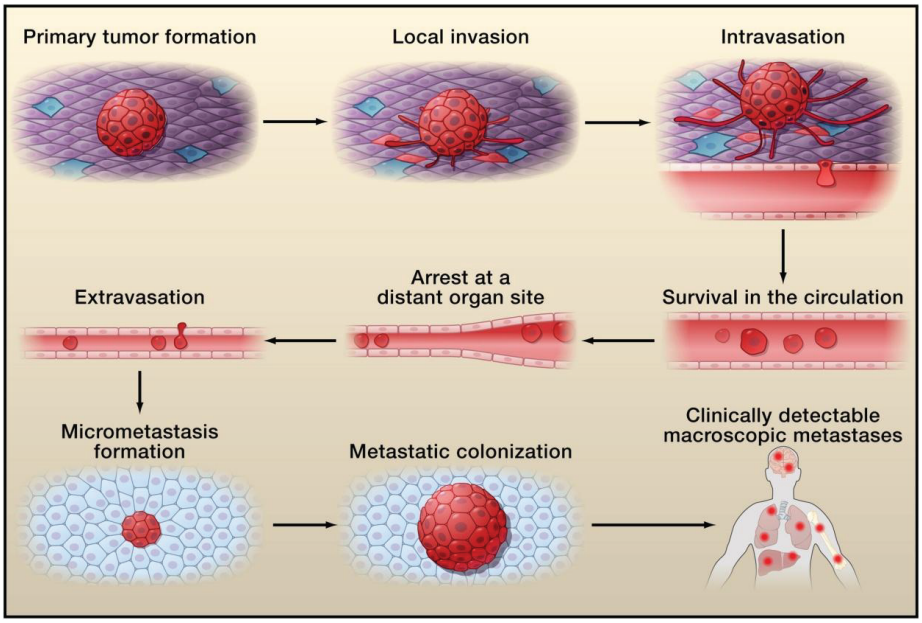

Figure 7: The process of invasion and metastasis. From (69), with permission.

A model for the capacity to invade surrounding stroma is the epithelial to mesenchymal transition (EMT), where cancer cells lose their adherent, epithelial properties and are converted to invasive, mesenchymal-like cells. Hypoxia is known to upregulate transcription factors that facilitate this transition, such as TWIST1 and SNA1, and enzymes that modify collagen fibers such as LOX (70). Interestingly, animal studies have shown that cyclic, but not chronic hypoxia, enhanced spontaneous lung metastasis in xenograft-bearing mice (71), but it is still an unresolved question whether cyclic or chronic hypoxia has the strongest influence on the metastatic phenotype (72, 73).

2.2 Transcriptional regulation of cancer hallmarks

The central dogma of molecular biology states that DNA is transcribed to RNA which is translated to protein (74). An important regulatory step in this pathway is control of gene transcription, i.e. the decision of which parts of DNA that shall be transcribed to RNA, and when this should occur. All somatic cells in the body contain the same DNA, thus, transcriptional regulation forms the basis of differentiated cellular function and adaptation of cells and organisms to different environments and demands (19). Some of the genomic alterations in cancer lead to aberrant gene expression and regulation, which drive development of cancer hallmarks and tumor progression.

The introduction of global gene expression analysis by microarray techniques and, recently, RNA sequencing has revealed novel insight into how the cancer hallmarks associate with specific changes in the transcriptional program. The use of these techniques for analysis at the DNA level has shown that cancers are highly heterogeneous at the genomic level. However, recently Markert and co-workers analyzed a set of gene expression profiles and revealed two common prognostic transcriptional programs across several types of cancers, including prostate cancer, i.e. proliferation and tissue remodeling (16). The tissue remodeling transcriptional program was related to hypoxia and associated with HIF1 activation (16). In this thesis, we focused on regulation of gene expression by gene regulatory proteins and epigenetic mechanisms through histone acetylation status. In addition, the transcriptional program in prostate cancer was examined on a whole-genome basis in relation to hypoxia and the proliferation marker Ki67.

2.2.1 Gene regulatory proteins

Gene regulatory proteins, hereafter referred to as transcription factors, bind to DNA in the promotor region of their target genes and hereby affect the amount of transcription, working either as transcription activators or repressors (19). A single transcription factor has the ability to affect transcription of several genes. Conversely, the transcription of a single gene may be influenced by several transcription factors (75). A transcription factor of particular importance for the pathogenesis of prostate cancer is AR, whereas a transcription factor that has been shown to play a major role in the regulation of gene expression under hypoxia is HIF1.

The AR transcription factor

The gene for AR is located on the X-chromosome and is widely expressed in both sexes, especially in reproductive tissues. Its main ligand is dihydrotestosterone (DHT), which is the

product of intracellular conversion of testosterone by 5alpha-reductase (23). Testosterone, a steroid hormone synthesized from cholesterol in the testicular Leydig cells, is transported in the blood through binding proteins like sex hormone binding globulin (SHBG).

The AR-ligand complex binds to androgen-responsive elements in the DNA and alters the transcriptional program (Figure 8, (23)). In addition to proliferation and cell cycle progression, androgen dependent gene expression induce metabolism, which provide energy and availability of biomolecules for prostate cancer carcinogenesis (50). The importance of AR in prostate cancer is exemplified by the effect of androgen deprivation therapy (ADT) on prostate cancer survival and metabolism, as further discussed in section 2.3.7 under palliative treatment modalities for prostate cancer.

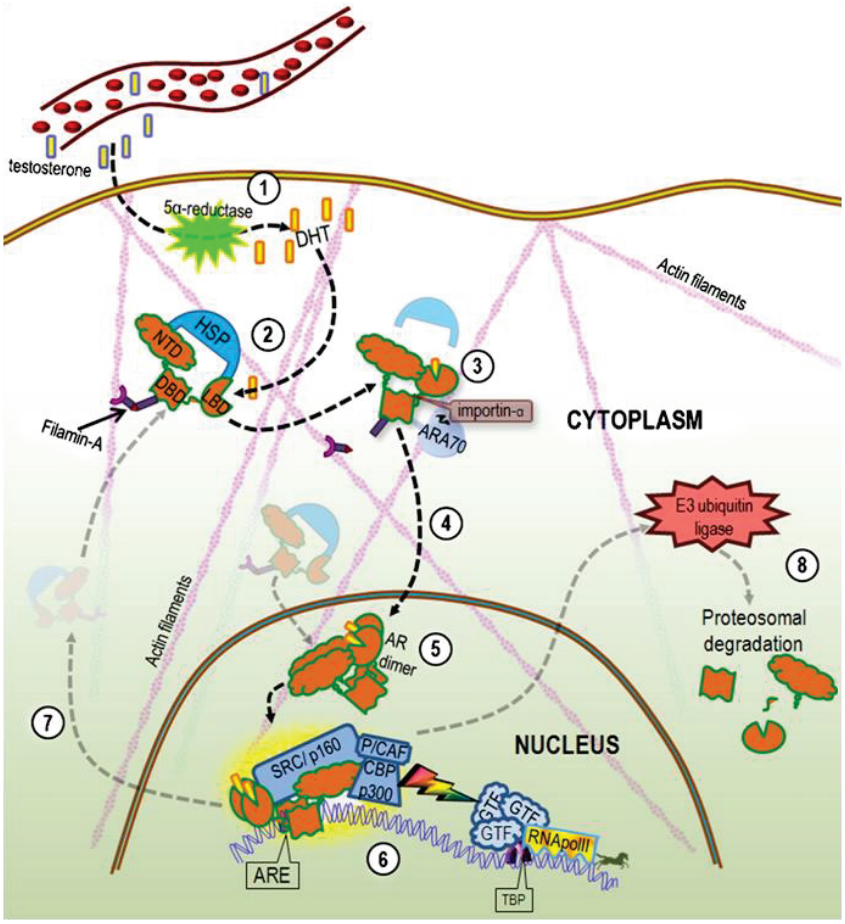

Figure 8: AR with ligands and co-regulators. The numbers refer to the figure: Testosterone is converted to dihydrotestosterone by 5-alpha-reductase (DHT; 1) and binds to AR (2) at the ligand-binding domain (LBD). This leads to conformational change of the receptor, dissociation of the heat shock protein (HSP) and recruitment of proteins important for stabilization and translocation to the nucleus (importin-α, ARA 70, Filamin-A; 3, 4). Following dimerization (5), several co-regulators bind to the AR at the N-terminal transactivation domain (NTD; 6). The receptor binds to androgen responsive elements (ARE) through the DNA binding domain (DBD), which promote recruitment of proteins with histone acetyltransferase activity (CBP/p300, P/CAF, SRC/p160), resulting in chromatin remodeling and facilitating transcription through TATA binding protein (TBP) and general transcription factors (GTF). Non-ligand bound AR can either be shuttled back to the cytoplasm and re-cycled (7) or be targeted for proteosomal degradation following ubiquitylation by E3 ubiquitin ligase (8). Figure and legend adapted from (23), with permission.

The HIF1 transcription factor

The hypoxia inducible factors are a set of proteins involved in numerous biological processes. The best characterized is HIF1, which consists of two subunits, the oxygen regulated HIF1α and the constitutively expressed HIF1β (ARNT) (76). The expression and transactivating capabilities of HIF1 is regulated by both oxygen-dependent and -independent mechanisms (Fig. 9, 10; (38)). In normoxia prolyl hydroxlase (PHD) will contribute to oxygen dependent hydroxylation of HIF1α, which results in binding of the von Hippel-Lindau (VHL) protein to HIF1α, followed by degradation. Under hypoxia will the degradation of HIF1α not occur and it dimerizes with HIF1β (Fig. 10). The HIF1 complex translocates to the nucleus and binds to hypoxia-responsive elements (HREs) on the DNA strand, which leads to alterations of the transcriptional program, as shown in Figure 10 and 11.

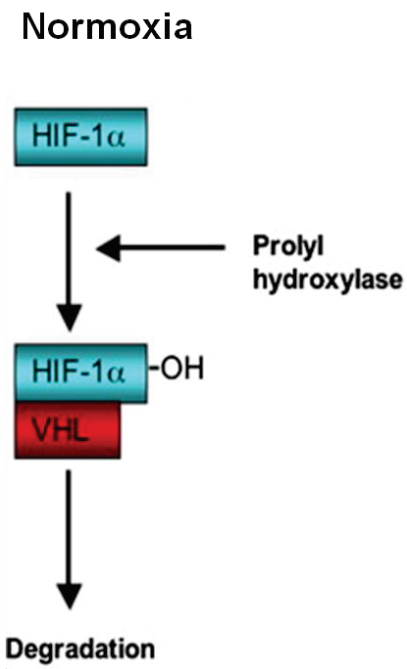

Figure 9: HIF1α degradation under normoxia. See text for details. Figure adapted from (38), with permission.

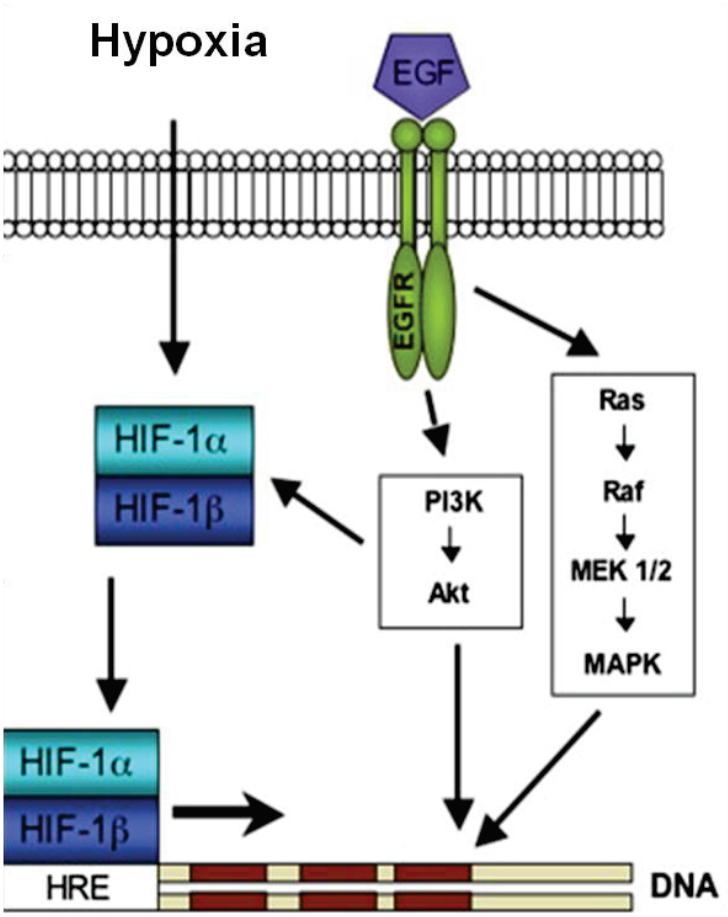

Figure 10: HIF1α regulation. See text for details. Figure modified from (38), with permission.

Oxygen independent regulation of HIF1 includes pathways that stimulate proliferation such as the PI3K-AKT and RAS-RAF-MEK-MAPK signaling pathways (Figure 10; (77)). In prostate cancer a cross-talk between HIF1 and AR has been investigated. Mabjeesh and co-workers found that activation of AR by androgens led to increased secretion of epithelial growth factor (EGF) and autocrine binding of EGF to EGF receptor (EGFR), which in turn stimulated the PI3K-AKT pathway and transcription of HIF1α (Figure 10, (78)). With the pivotal role of AR function in mind, this finding suggests that oxygen independent mechanisms can be important in prostate cancer.

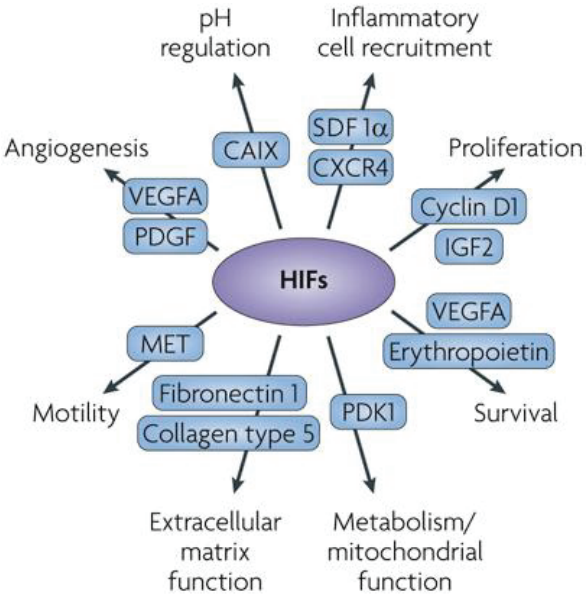

Figure 11: Biological processes and protein expression altered by HIFs. Abbreviated names: CAIX, carbonic anhydrase IX; CXCR4, chemokine (C-X-C motif) receptor 4; IGF2, insulin-like growth factor 2; PDGF, platelet-derived growth factor; PDK1, pyruvate dehydrogenase kinase, isoenzyme 1; SDF1α, stromal cell-derived factor 1α; VEGFA, vascular endothelial growth factor A. Figure reproduced from (37), with permission.

The alterations of biological processes by HIF1, for instance induction of angiogenesis and regulation of pH in response to anaerobic metabolism (Figure 11), is a part of the cellular stress response to hypoxia (37). At the same time, genes encoded by HIFs are involved in several processes important for tumor initiation, growth, invasion and metastasis, such as modulation of extracellular matrix, motility, proliferation and inflammatory cell recruitment (37), creating associations to several hallmarks of cancer (12). Expression of HIF1α has been observed in several cancers, including prostate cancer (6, 79-81), and Zhong and co-workers demonstrated an upregulation of HIF1α in prostatic intraepithelial neoplasia as compared to adjacent normal glands, pointing to a possible function in early prostate carcinogenesis (81). Moreover, high HIF1α expression has been shown to associate with poor outcome of prostate cancer patients after both surgery and radiotherapy (80). HIF1 therefore seems to be a transcription factor of high importance in prostate cancer (82)

2.2.2 Epigenetic modification of DNA by changing histone acetylation status

The primary unit of chromatin is the nucleosome, which consists of DNA wrapped around a histone complex. The histones can be target of chemical modification, in form of acetylation, methylation and phosphorylation of the N-terminal ends, which are all important epigenetic regulatory mechanisms for gene transcription. Histones with acetylated lysine residues are in general associated with a looser chromatin conformation, which allows access to DNA by transcription factors and RNA polymerase, thus enhancing gene expression (83). Histone acetylation status is a reversible process where enzymes within the classes histone acetyltransferase (HAT) and histone deacetylase (HDAC) have opposing actions. The balance between these enzymes regulates expression of many genes, and HDACs have also been shown to modulate non-histone protein function (Figure 12; (83)).

Anti-cancer activity of HDAC inhibitors has been investigated in many cancer entities both as monotherapy and in combination with chemotherapy and radiation (84). Vorinostat, also called suberanilohydroxamic acid or SAHA, inhibits HDAC function through zinc chelation and is approved for treatment of cutaneous T cell lymphoma. In addition, the drug has shown anti-proliferative and apoptotic effects *in vitro* and growth suppressive effects *in vivo* in prostate cancer models (85). Vorinostat influences the expression of various genes and proteins (Figure 12), and the precise mechanism of action leading to apoptosis, differentiation and radiosensitization of cancer cells is not fully elucidated. However, several studies indicate that HDAC inhibitors target the oncogenic action of HIF1 (86).

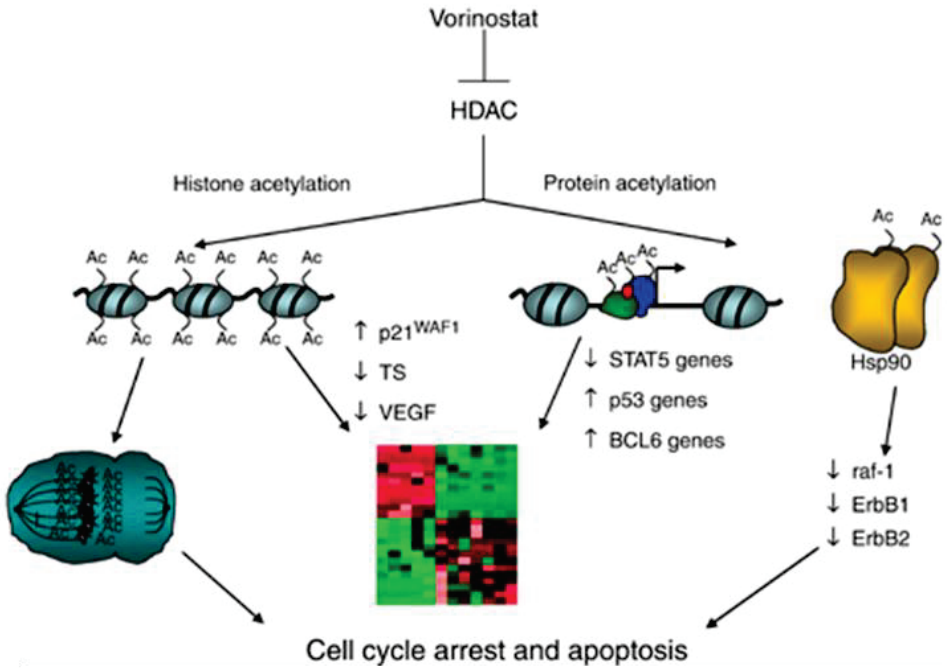

Figure 12: Proposed action of vorinostat leading to cell cycle arrest and apoptosis. Vorinostat inhibits HDAC activity which leads to increased acetylation of histones and other proteins. This affects the chromatin structure, transcription factors, the mitotic spindle apparatus and other regulatory proteins such as the chaperone heat shock protein 90 (HSP90). Adapted from (87), with permission.

Vorinostat as radiosensitizer

The limiting factor in radiotherapy is the sensitivity of the surrounding normal tissues and thereby the risk of radiation induced morbidity (88). Thus, strategies to improve the radiosensitivity of tumor cells without sensitizing normal cells are highly warranted (88). As discussed above in section 2.1.2, tumor hypoxia increases the radioresistance and constitutes a therapeutic problem in radiotherapy. Treatment with radiosensitizing drugs showing efficacy in hypoxic cells could be an option to overcome this problem. Chinnaiyan and co-workers found a radiosensitizing effect of vorinostat in the prostate cancer cell line DU 145 under normoxia. Mechanistically, concurrent vorinostat and radiation treatment reduced the induction of DNA damage proteins RAD51 and DNA-PK, and increased apoptosis as compared to radiation alone (89). Recently, a phase 1 trial combining vorinostat with palliative pelvic radiotherapy for metastatic colorectal cancer was conducted at our institution

(90). In addition, we have shown radiosensitizing effects of vorinostat under normoxic and hypoxic conditions in a colorectal cancer xenograft model (91, 92). Vorinostat is therefore a candidate for combating hypoxia induced radioresistance in prostate cancer, and was further examined in this thesis.

2.3 Prostate cancer

2.3.1 Prostate anatomy and physiology

The prostate is an exocrine gland and a part of the male accessory sexual organs. It is located below the urinary bladder and surrounds the urethra (Figure 13). The function of the prostate is to produce an alkaline fluid involved in maturing and nourishing sperm and in dissolving the cervical plug. The prostate can be divided into an anterior fibro-muscular stroma, in addition to three glandular zones: The peripheral, transitional and central zones (93, 94). Two of the main disease entities in the prostate are benign prostatic hyperplasia and prostate cancer. Approximately 70 % of prostate cancers arises in the peripheral zone (95).

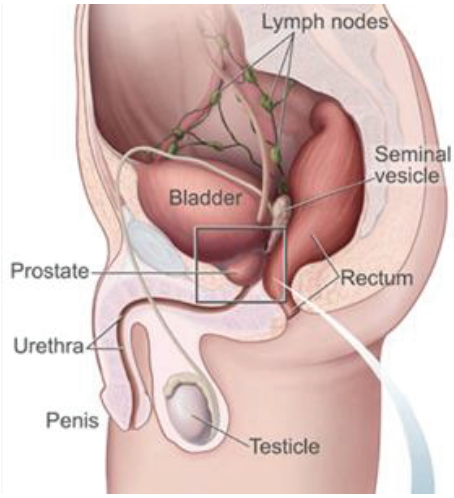

Figure 13. The prostate with surrounding structures. From Wikipedia.

2.3.2 Epidemiology and etiology

In Norway, 4978 men were diagnosed and 1052 men died from prostate cancer in 2011, making the disease the most commonly diagnosed and the second leading cause of cancer-related death among males (1). Correspondingly, prostate cancer is the second most

commonly diagnosed, and the sixth leading cause of cancer-related deaths among males in the world (2). Studies of men from USA show that African-Americans have higher incidence and mortality from prostate cancer, and men of Asian ethnicity have lower incidence and mortality compared to Caucasian men (96). Prostate cancer is primarily a disease of the elderly men, with a median age at diagnosis of 72 years in Norway in 2004 (97). The mortality has declined since the 1990s, which has been attributed to improved treatment and possibly the use of the PSA test (2).

Lifestyle has been discussed as a possible risk factor of developing prostate cancer, with dietary factors such as obesity and high fat consumption as risks, and intake of soy and vegetables such as tomatoes being protective (98). The only established etiological factors, however, are age, family history and ethnicity (99, 100). The association between age and prostate cancer is not only found in the clinic, but also in autopsy studies where the prevalence increases with higher age (101).

First-degree relatives of prostate cancer patients diagnosed before the age of 60 have more than four times the risk of acquiring prostate cancer compared to men without family history (99). The contribution of germline genetic alterations is largely unknown, but some gene aberrations have been identified as risk carriers, such as mutations in the tumor suppressor genes *BRCA1* or *BRCA2*, which has been found to elicit approximately four and five times higher risk of prostate cancer compared to wild-type, respectively (99). A recurrent somatic mutation is the fusion between *TMPRSS2* and members of the *ETS* gene family, with the most frequent aberration being the *TMPRRS2-ERG* fusion gene (102).

2.3.3 Carcinogenesis, morphology and grading

The vast majority of prostate cancers have differentiation pattern as adenocarcinoma (103). The only accepted precursor is high-grade prostatic intraepithelial neoplasia (HG-PIN), which is characterized by hyperplasia of glands, but with retained basal cell layer and without invasive properties (104). Prostatic inflammatory atrophy, i.e. atrophic areas where cells regenerate in response to unknown cellular damage (105), has also been linked to prostate cancer development (106).

The dominating grading system for prostate cancer in the world today is the Gleason score, originally presented by the American pathologist Donald Gleason in 1966, with the latest consensus revision performed in 2005 (107). A prostate tumor is assigned a Gleason score based on the morphological architecture of the cancerous glands. The score is the sum of the two most prevalent Gleason grades in the specimen, where each grade is a number

between 1 and 5. A higher score is associated with a more aggressive tumor, and a tumor with high Gleason score is in general characterized by poor differentiation, fused glands and necrotic areas (107). Prostate cancer is often multi-focal, with different Gleason scores within one specimen. There has been international consensus-meetings with goal of defining the index or dominating tumor among these (108). In our work, the index tumor was defined as, in descending order, the tumor which gave highest pT category, Gleason score or tumor size.

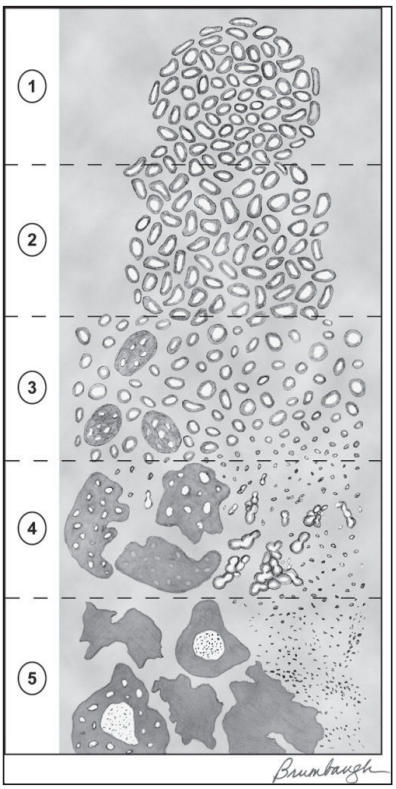

Figure 14. The Gleason grading system for prostate cancer. From (107), with permission

2.3.4 Clinical presentation, PSA testing and diagnosis

Localized prostate cancers are in the vast majority asymptomatic, but symptoms such as nocturia, hesitancy and obstruction may be present, often due to concurrent benign prostatic hyperplasia (109). Metastatic disease can lead to bone pain, neurological symptoms from compression of the spinal cord and anemia (110). Prostate cancer is diagnosed by microscopy of core biopsies taken ultrasound-guided, with transrectal or transperineal approach, or of resected material following a transurethral resection of the prostate (TUR-P) or

cystoprostatectomy. MRI images may be of help in evaluating the risk of extra-prostatic cancer (111).

PSA (official name: kallikrein-related peptidase 3) is a serine protease produced by prostate epithelium. PSA liquefies the semen and dissolves the cervical plug, hereby helping the sperm to swim freely. As the name implies, the production of PSA is highly specific to the prostate gland (112). A small amount of the produced PSA escapes into the blood circulation, and is subject to concentration measurements clinically. However, moderately elevated PSA levels are not specific to prostate cancer, but occur also as a result of benign prostatic hyperplasia and prostatitis (100). The normal range of PSA increases with higher age. It is difficult to set a definitive cut-off value, but levels below 3-4 ng/mL are often considered normal, although patients with aggressive cancer may have lower levels (100).

The PSA test has been evaluated for prostate cancer screening, where patients without symptoms are tested to detect disease at an early stage in order to reduce mortality and improve future quality of life (113). In a European multicenter study, a reduction in prostate cancer mortality through PSA screening was demonstrated (3, 114). In an American study however, no statistically significant benefit on mortality was observed (115). In 2012, the Cochrane collaboration issued a review of the literature where they concluded that prostate cancer screening, defined as PSA testing with or without digital rectal examination, gave no significantly reduction in prostate-cancer specific mortality in an unsorted population (113). This is in line with conclusions from the U.S. Preventive Services Task Force (116), and Norwegian health authorities advises against PSA testing in an unsorted population (117). However, for patients with risk factors such as African-American heritage or family history with frequent diagnosis of prostate cancer and/or known mutations in susceptibility genes such as *BRCA1 and BRCA2*, the value of PSA testing potentially increases (116, 117).

2.3.5 Staging and risk classification

The disease stage is given by the tumor, node, metastasis (TNM) system (118). A description of the clinical TNM system is presented here: Tumor stages TX and T0 reflect a primary tumor that is either not possible to assess or find, respectively. Stage T1 indicates that the primary tumor is not palpable, but cancer is found by microscopy of tissue, either in material from TUR-P (T1a-b) or in a core biopsy after PSA testing (T1c). Stage T2 denotes a tumor that is palpable, but confined to the prostatic gland. In T2a less than half of one lobe is infiltrated, in T2b more than half one lobe is infiltrated, and in T2c both lobes are infiltrated (Figure 15). Stage T3a signifies a tumor growing out of the prostatic capsule, and T3b a tumor

growing into the seminal vesicles (Figure 15). In stage T4 the tumor has grown into adjacent organs such as the pelvis or bladder (Figure 15).

All patients with lymph node metastases (N1) are high-risk patients, and there is controversy as to whether these patients are truly curable, despite pelvic lymphadenectomy (100). Patients with distant metastasis to non-regional lymph nodes or other sites are assigned M1 and are not candidates for curatively intended therapy (100).

The gold standard for lymph node staging is pelvic lymphadenectomy, but magnetic resonance imaging (MRI) and computerized tomography (CT) may also be performed, albeit with lack of diagnostic accuracy (119). The use of risk nomograms has made pelvic lymphadenectomy superfluous prior to definitive therapy for patients at low risk of lymph node metastases (120). For M-staging, technetium scintigraphy is recommended in patients with PSA values above 20 ng/mL, or with Gleason score above 7a and clinical T-stage above 2 (100).

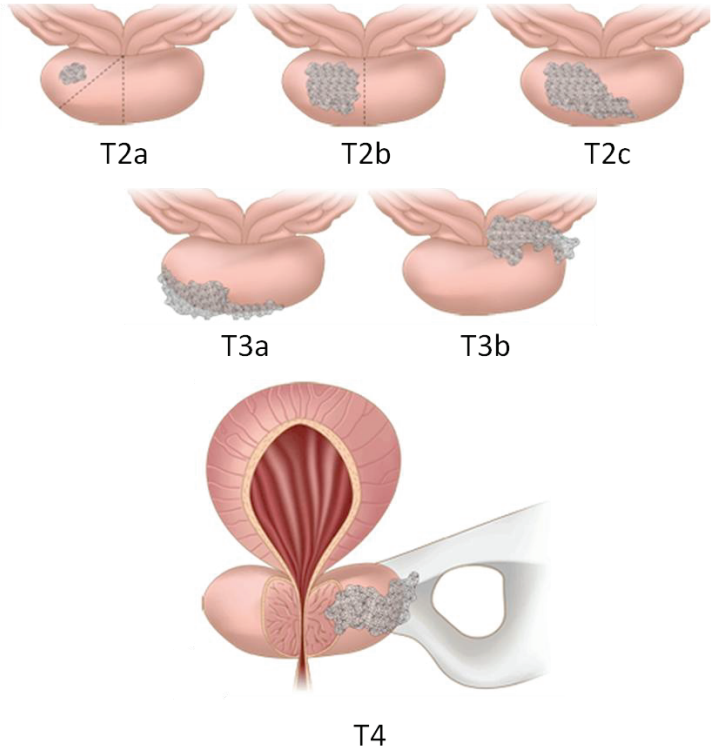

Figure 15: T-stages of prostate cancer. Adapted from (121), with permission.

Some patients diagnosed with prostate cancer will, due to indolent disease, never experience symptoms or disease progression, whereas others will have a rapid progression (122). The heterogeneity in disease aggressiveness prompts the need for reliable markers for correct management of prostate cancer patients (122). In general, low-risk patients could be candidates for surveillance and deferred treatment, whereas high-risk patients are candidates for combination of treatment modalities (100), as discussed below.

Current risk estimation relies on clinicopathological parameters from studies of patients undergoing definite therapy in form of surgery or irradiation, where time to biochemical failure, defined as recurrence of PSA, was recorded (100). One of the most widely used risk classifications is the D'Amico risk model, based on Gleason score, PSA-level and clinical T-stage at diagnosis (123). Another risk model is the University of California, San Francisco-Cancer of the Prostate Risk Assessment (UCSF-CAPRA) score, where, in addition to the above mentioned parameters, percentage of positive core biopsies and patient age are included in the risk model (124).

Methods for prostate cancer risk classification that have not yet reached the clinic include DNA, RNA and protein based biomarkers. A candidate DNA-based prognostic marker is the fusion gene *TMPRRS2-ERG,* which has shown variable independent prognostic value in different cohorts (125). Several RNA expression signatures have been developed for prognostication, but they are in general hampered by small cohort sizes and lack of independent validation (125). Moreover, gene signatures that show independent prognostic information have little overlap (126).

Ki-67 is a protein biomarker that can be detected by immunohistochemistry. The protein is expressed in interphase cells, and Ki-67 is therefore a marker of proliferation. Ki-67 staining of cancer tissue has been shown to add independent prognostic value in several prostate cancer cohorts, but due to factors like different cut-off values for positivity, it has still not reached clinical decision making (125). Other protein biomarkers that have been investigated, but not reached the clinic, include cell-cycle related proteins such as CDKN1A (p21) and apoptosis related markers such as BAX (127).

2.3.6 Curative treatment modalities

Choice of prostate cancer treatment modality relies on the TNM stage, age and co-morbidity, Gleason score, PSA-level and the patient's own wishes. Two main curative treatment options are available in this category: Surgery and radiotherapy. The choice between these two is

highly controversial, as there are no randomized trials comparing them. Typically, a young and fit patient with localized disease will often be referred to surgery, while an older patient with locally-advanced disease and co-morbidity will be subjected to radiotherapy. There are however many exceptions from this rule.

Long-term side-effects of both radiotherapy and surgery include impotence and incontinence. In addition, radiotherapy can induce rectal toxicity with diarrhea and bleeding, and surgery carries perioperative risks. Hence, a treatment form gaining ground the last years for low risk patients is active surveillance. Patients on active surveillance programs are followed closely with repeated digital rectal examinations, PSA measurements and biopsies, and definite treatment is deferred until signs of progression occur, but at a disease stage where cure is still possible (100).

Surgical treatment of prostate cancer can be performed as open retropubic prostatectomy (ORP) or as robot-assisted laparoscopic prostatectomy (RALP). ORP has shown disease-specific and overall survival benefit in localized prostate cancer in one randomized trial as compared to watchful waiting (128), but this was not shown in another study (129). The first RALP was performed in 2000, and has since replaced more and more of the ORPs, also in Norway. RALP has shown to reduce peri-operative bleeding and incidence of blood transfusion, with comparable outcome as ORP (130). For high-risk prostate cancer patients, and where lymph node metastases are detected by imaging, prostatectomy combined with lymph node dissection should be performed, as this has been shown to prolong survival (100, 131).

Radiotherapy for prostate cancer can be applied in several ways. The EAU guidelines recommend intensity-modified external beam radiotherapy (IMRT) with or without image-guidance, combined with ADT for D'Amico intermediate and high-risk patients (100). Other treatment regimes include brachytherapy with low- or high-dose rate irradiation, alone or in combination with external beam radiotherapy and/or ADT.

2.3.7 Palliative treatment modalities

Palliative treatment forms can be offered patients that are no longer candidates for curative treatment, due to advanced disease stage on presentation or because of a watchful waiting strategy. Watchful waiting is another form of deferred treatment suitable in patients where cure is not the goal, due to factors like old age or co-morbidity. Any treatment is usually put on hold until signs of disease progression occurs (100).

A peculiarity of prostate cancer is the androgen dependence of the disease, described by Huggins and Hodges in 1941 (132). Since Huggins and Hodges work it has become evident that AR activity is important for viability, metabolism, proliferation and invasion of prostate cancer cells (50, 133, 134). As such, the backbone of palliative treatment is ADT, where the hypothalamus-pituitary-testicular axis is manipulated.

One ADT approach is to perform bilateral orchiectomy, leaving the adrenal cortex alone in producing androgens. This is the most rapid way of reducing the testosterone level, and therefore remains the preferred treatment where a rapid reduction is wanted, as in spinal cord compression. Less drastic and irreversible methods are present however, in form of gonadotropin-releasing hormone (GnRH) agonists and antagonists, as well as steroidal and non-steroidal anti-androgens (100). GnRH agonists and antagonists will inhibit a stimulatory effect from the hypothalamus on the pituitary gland, thus reducing the level of luteinizing hormone and thereby decreasing the testosterone production by the testis. The GnRH agonists will however cause an initial flare-up period of higher testosterone levels, which could complicate a clinical situation of for instance skeletal pain. These complications can be counteracted by initially including the use of a non-steroidal anti-androgen, which are drugs that block the AR, thus interfering with androgen signaling (135).

Eventually, the cancer will turn castration-resistant, i.e. progress despite ADT. In recent years new treatment modalities in form of immunotherapy, radio-immunotherapy and second-line endocrine treatments have become available for castration-resistant disease, in addition to standard chemotherapy like docetaxel. The improvement in life-expectancy by these treatment modalities is however limited, and new strategies are highly warranted (100).

3. Summary of papers

Paper I: *The Hypoxia Marker Pimonidazole Reflects a Transcriptional Program Associated with Aggressive Prostate Cancer*

In Paper I we used pimonidazole immunohistochemistry and whole-genome gene expression analysis to identify transcriptional changes in hypoxic prostate cancer. We utilized material from 46 prostate cancer patients included in the clinical protocol, which was established as one of the thesis aims. Transcriptional changes associated with pimonidazole staining showed enrichment of the biological processes cell cycle, translation and cellular response to stress. We performed supervised gene set analysis with input gene lists encompassing these processes, in addition to lists of hypoxia-associated genes. A gene signature of 32 genes, all with a positive correlation to pimonidazole staining, was constructed. Nine of these genes were HIF1 targets.

Pimonidazole staining was associated with aggressive disease, demonstrated by increased presence of clinical stage T3 and lymph node metastasis in patients with high immunoscore. The pimonidazole gene signature was also associated with aggressive disease, in form of higher clinical T-stage and Gleason score and presence of lymph node metastasis in patients with high gene score. In addition, unsupervised clustering of the investigation cohort based on the pimonidazole gene signature showed association to presence of lymph node metastasis. Furthermore, expression of the pimonidazole-associated genes showed a positive correlation to the proliferation marker Ki67.

To validate the association between our gene signature and aggressiveness, we procured two gene expression data sets from prostate cancer cohorts with information on Gleason score in the first, and survival in the second validation cohort. High expression of the pimonidazole gene signature was associated with Gleason score above 7a. Moreover, high expression was associated with reduced progression-free and overall survival in both uni- and multivariate analysis, the latter corrected for known aggressiveness markers such as Gleason score and *TMPRSS2-ERG* translocation.

In conclusion, pimonidazole staining and associated transcriptional changes were related to an aggressive prostate cancer phenotype, including increased proliferation, as measured by Ki67. This may imply that genes involved in proliferation, DNA repair and hypoxia response contribute to prostate cancer aggressiveness, and that expression of these genes has the potential of being used as a prognostic biomarker.

Paper II: *Hypoxia-independent Downregulation of Hypoxia-Inducible Factor 1 Targets by Androgen Deprivation Therapy in Prostate Cancer*

Since hypoxia is a risk factor in prostate cancer radiotherapy, a biomarker for tissue hypoxia during neo-adjuvant ADT would be helpful in timing of radiotherapy. To evaluate the use of HIF1α expression in this context, we analyzed HIF1α expression and gene transcription downstream of HIF1 in androgen exposed and androgen deprived CWR22 prostate cancer xenografts. In a previous study we had demonstrated no statistically significant differences in pimonidazole staining between these two conditions, which implied that the hypoxic fraction was comparable. To examine the clinical relevance of our findings, we also included HIF1α immunohistochemistry of a prostate cancer cohort subjected to ADT prior to radiotherapy.

Whole-genome gene expression analysis revealed 1344 genes with more than two-fold expression change by ADT. Gene ontology analysis of these showed enrichment of the biological processes cell cycle, cellular response to stress, response to organic substance, steroid metabolic process, cell proliferation, monosaccharide metabolic process and apoptosis. Comparison of the differentially expressed genes with published lists of HIF1 targets, revealed that thirty-five downregulated and five upregulated HIF1 target genes were affected by ADT. The majority of HIF1 targets with transcriptional change by ADT were induced by hypoxia in androgen-responsive prostate cancer cell lines, supporting their role also in prostate cancer. Nineteen downregulated HIF1 targets were involved in the significant biological processes, most of them in energy metabolism. Four of these were shared AR and HIF1 targets, including genes encoding the regulatory glycolytic enzymes HK2, PFKFB3 and SLC2A1 (GLUT1).

The downregulation of HIF1 targets by ADT was consistent with reduced HIF1α expression in the androgen exposed xenografts, despite comparable hypoxic fractions. In addition, downregulation of HIF1α protein was seen in patient tumors going from androgen exposed to androgen deprived state.

In conclusion, ADT may lead to decreased HIF1α protein expression irrespective of changes in hypoxic fraction. This was accompanied by downregulation of HIF1 signaling, including HIF1 target genes involved in the biological processes enriched by ADT, which suggests that HIF1 plays a role in the regressive phase under androgen deprivation. In this context, HIF1α downregulation seems to be a marker for androgen deprivation per se, rather than reduced hypoxic fraction.

Paper III: *Vorinostat-Mediated Radiosensitization and Transcriptional Effects in Hypoxia-Treated Prostate Cancer Cell Lines*

In Paper III we analyzed radiosensitizing effects of the HDAC inhibitor vorinostat on prostate cancer cell lines under hypoxic and normoxic conditions, and related these effects to changes in gene expression. We chose the DU 145, PC-3 and 22Rv1 prostate cancer cell lines since they had shown differential sensitivity to radiation and vorinostat.

Following vorinostat exposure, a radiosensitizing effect was observed under normoxia and hypoxia in DU 145, at both 2 Gy and 5 Gy irradiation. Conversely, PC-3 showed only a trend towards radiosensitization. Vorinostat had radiosensitizing effects in 22Rv1 that reached statistical significance at 2 Gy under normoxic and 5 Gy under hypoxic conditions. Moreover, DU 145 showed a statistically significant accumulation of cells in G_2/M phase, which may have contributed to the radiosensitizing effect. Significant changes in cell cycle distribution were not observed in PC-3. In 22Rv1, an accumulation of cells in G_1 under both normoxia and hypoxia was observed, but this experiment lacked replicates, making the result uncertain. Hypoxia treatment in itself had no significant effects on the cell cycle distributions.

To evaluate transcriptional effects by vorinostat, we selected DU 145 and PC-3, which showed the largest difference in radiosensitization. Based on the gene expression data, we found that DU 145 showed enrichment of several biological processes, among them cell cycle and cellular response to stress. PC-3 had fewer enriched processes. In DU 145, a reduced expression of HIF1α was seen after vorinostat exposure under both normoxic and hypoxic conditions, whereas this was not observed in PC-3 or 22Rv1. To provide evidences that the reduced HIF1α protein level was followed by a change in HIF1 signaling, we performed a network analysis on HIF1 targets and their protein interactions, where the interaction partners were selected among the genes that showed significantly altered gene expression by vorinostat. DU 145 had larger interaction networks than PC-3, involving a higher number of both direct targets and interaction partners. The networks showed similarity between normoxia and hypoxia treatment classes, especially for DU 145.

In conclusion, vorinostat treatment of prostate cancer cell lines showed promising radiosensitizing properties under hypoxic and normoxic conditions, suggesting that vorinostat has a potential as a method to overcome hypoxia related radiation resistance in prostate cancers. We demonstrated changes in HIF1α protein expression and HIF1 transcriptional program after vorinostat treatment, and propose that HIF1α or its target genes may have a role as biomarkers for the radiosensitizing effects.

4. Evaluation of materials and methods

4.1 Clinical protocols

4.1.1 The Funcprost protocol

The clinical protocol established as part of this thesis is "MRI for assessment of hypoxia-induced prostate cancer aggressiveness", in short termed Funcprost (ClinicalTrials.gov: NCT01464216). The aim of the Funcprost study is to evaluate hypoxia driven prostate cancer aggressiveness, and in Paper I, some of the material from the study was analyzed. The Funcprost protocol encompasses work by several scientists and clinicians, and the author of this thesis had the role as study coordinator, which comprised practical organization of investigations, inclusion of patients, pimonidazole infusion, bone marrow aspiration and collection of cancer biopsies. The Funcprost study was approved by the regional ethics committee and the institutional board. Patients received a formal description of the study in lay language and gave signed consent to participation. We organized the protocol in accordance with the Norwegian act on medical and health research (the Health Research Act) from 2008. An unofficial English translation of the law is given by The Faculty of Law, University of Oslo (136).

The inclusion criterion in the Funcprost study was biopsy confirmed prostatic adenocarcinoma in a patient referred for surgery. In addition, patients should have intermediate or high risk disease according to D'Amico classification (123). These wide criteria caused inclusion of patients within a broad range in aggressiveness, which increased the dynamics in hypoxic fraction across the tumors, and the possibility of finding associations to biological and clinical parameters.

Exclusion criteria included previous cancer forms, contraindications to MRI contrast agents, allergies to substances chemically related to pimonidazole, for instance metronidazole, or previous treatment for prostate cancer such as radiation or hormonal manipulation. Bone marrow aspirates were performed to search for disseminated tumor cells at time of surgery. Due to this, we wanted to exclude patients with a previous cancer diagnosis, as the etiology of a positive test otherwise could be equivocal. Patients with history of previous prostate cancer treatments were excluded since we aimed to analyze molecular biology of prostate cancer unaffected by androgen deprivation or radiation therapy.

All patients underwent MRI examination within one week prior to surgery. The MRI protocol included functional sequences in form of diffusion weighted MRI, dynamic contrast

enhanced MRI and blood oxygen level dependent MRI (Fig. 16). In the work included in this thesis, the MRI images were used in prediction of index tumor localization to improve correct biobanking of biopsies. With guidance from these images, biobanking was performed with a punch biopsy knife after manual identification of cancer foci (Figure 16). We harvested two tumor biopsies from the suspected index tumor site, in addition to a biopsy from normal tissue. The use of a surgical cohort allowed us to evaluate the whole-mount section from the punch biopsy site, thus ensuring that we had taken the sample from the index tumor. This would not be possible in a radiotherapy cohort.

From October 2011 to July 2012 we included 53 prostate cancer patients referred for RALP at the Norwegian Radium Hospital, Oslo University Hospital. Unfortunately, we have not made a record of patients unwilling to participate, but this fraction is estimated to approximately 10 %. Of the 53 patients, 46 had fresh frozen punch biopsies which fulfilled our criteria for inclusion, and material from this group was analyzed in Paper I. Of the seven patients not included, one patient had postponed surgery, thus, biopsies were not taken. The remaining six patients had either biopsies not taken from the index tumor or index tumor biopsies that did not fulfill our criteria of at least 75% malignant glands and less than 50 % stroma.

Exclusion of patients with the lowest fraction of malignant glands may have enriched our investigation cohort for high tumor grades (107) and thereby probably reduced the variation in the biological data. Moreover, this led to less number of patients in the analysis and lower power in the statistical tests. Conversely, inclusion of biopsies with lower cancer cell fraction would have obscured the gene expression signal from tumor cells.

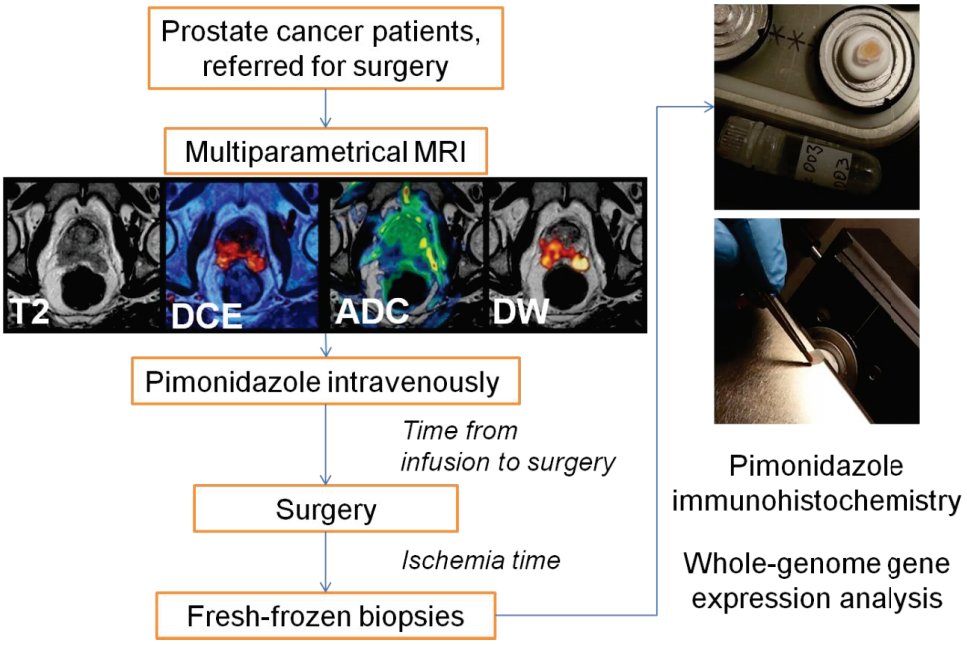

Figure 16: Flow diagram of investigations in the Funcprost project relevant for this thesis. MRI images courtesy of Knut Håkon Hole, MD, and printed with permission. *Abbreviations:* T2: T2 weighted image. DCE: Dynamic contrast-enhanced MRI with T2 image overlaid, ADC: Apparent diffusion coefficient with T2 image overlaid, DW: Diffusion-weighted image with T2 overlaid.

Pimonidazole infusion and time to surgery

Median time from pimonidazole infusion to total free dissection of the prostate was 19.4 hours with range 13.5-24.8 hours (Figure 16; Paper I). In the study protocol, planned time from infusion to surgery was 12-24 hours, which is close to what was achieved, and in line with other clinical studies, where time from infusion to surgery or biopsy typically has been 10-24 hours (137) or 16-24 hours (138, 139). For the patients included in Paper I, pimonidazole was available only for intravenous administration demanding access to infusion devises. Thus, the time points for pimonidazole administration were limited, and we were only able to administer pimonidazole to 43 of the 53 patients in the cohort. This corresponded to 39 of the 46 patients included in Paper I. In recent months however, pimonidazole has become available in oral formulations, facilitating adaptation of drug administration to time of surgery the following day. This does however demand a comparison of staining intensities

between the two methods before possible pooling of data. Hence, we chose to analyze the transcriptional program reflected by pimonidazole concurrently to the change from intravenous to oral administration.

With data available for 32 patients, we found no statistically significant correlation between pimonidazole immunoscore and time from pimonidazole infusion to surgery (r=-0.20; *P*=0.26), showing that the difference in time to surgery among the patients was not large enough to influence the score significantly (Paper I). Considering the limited power due to a low number of patients, we cannot, however, exclude that a long time from infusion to surgery could lead to lower pimonidazole score.

Ischemia time

The gene expression analysis in Paper I was based on material from fresh frozen tumor biopsies (Fig. 16). During surgery, the prostate will be gradually dissected free from the vasculature and subsequently placed in a bag inside the abdominal cavity, while the anastomosis between the bladder and the urethra is made. Loss of oxygen supply with following cellular reactions could pose a problem for the gene expression analysis. Consequently, it was a goal to keep time from total free dissection to snap-freezing as short as possible. For 36 of the patients, this time period; i.e. ischemia time, was recorded, showing range from 37-70 minutes and median 50 minutes (Figure 16; Paper I).

We searched for correlation between the pimonidazole gene score and ischemia time, and for differences in ischemia time between patients belonging to two different gene expression clusters. None of these correlation analyses showed any relation between ischemia time and gene expression data (Paper I). This is in accordance with previous work by Dash and co-workers, who studied changes in global gene expression in prostate cancer relative to ischemia time after radical prostatectomy (140). They found that only 0.6% of the investigated genes showed significant alterations in gene expression after one hour ischemia time before snap-freezing (140). Similar results have been obtained in breast cancer, where only a small fraction of genes had significant expression changes after the first hour (141). As such, with the aim of comparing gene expression between patients, we concluded that a median ischemia time of 50 minutes with range 37-70 minutes was acceptable.

Punch biopsies versus whole mount prostatectomy sections

Our protocol enabled analysis of pimonidazole staining in whole mount prostate sections and punch biopsies from the same patients (Figure 16). In identification of a transcriptional program represented by pimonidazole, we used the immunoscore from the punch biopsies to ensure full representativeness. For further analysis in the Funcprost protocol, such as pimonidazole in relation to MRI parameters and bone marrow micrometastases, it is important that the pimonidazole score in the punch biopsies reflects staining of the entire index tumor, represented by the whole mount section.

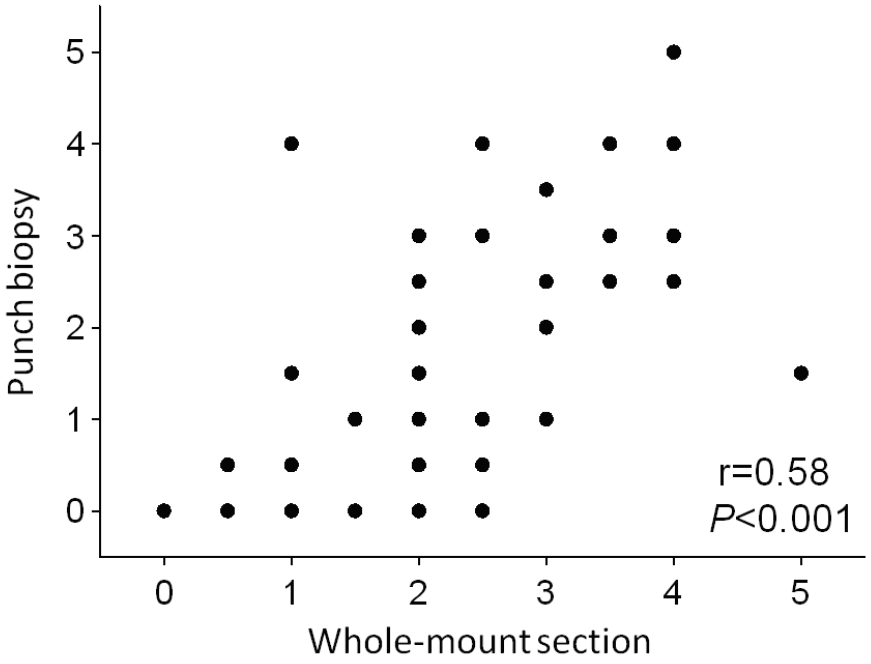

Figure 17: Pimonidazole immunoscores in punch biopsies versus whole mount sections. N=39, with 6 dots identical between the punch biopsies and the whole mount sections.

The statistically significant association between pimonidazole immunoscores of the punch biopsies and whole mount sections was 0.58 (Figure 17). As such, it seemed that the hypoxic status in the punch biopsies to a large degree reflected the hypoxic status in the whole mount sections. However, the association between punch biopsy and whole mount pimonidazole immunostaining is not perfect. Thus, there is intra-tumor heterogeneity in pimonidazole staining and variation in hypoxia in different parts of the tumor. This enables

future analysis of molecular alterations in different sub-areas of the same tumor showing different pimonidazole staining intensities.

4.1.2 The ADT protocol

In Paper II material from a clinical protocol on patients receiving conformal radiotherapy for prostate cancer was utilized (Figure 18). As part of the protocol, all patients received neo-adjuvant ADT consisting of goserelin acetate and bicalutamide 3-6 months prior to radiation. In preparation for radiotherapy, two biopsies from the prostate were taken with trans-rectal approach. In addition, we summoned the diagnostic, androgen exposed biopsies from the referring hospitals (Figure 18). This allowed us to compare the expression of HIF1α in androgen exposed (AE biopsy) and androgen deprived (AD biopsy) tumors from the same patients. For analysis, we used the needle biopsy with highest Gleason score from the androgen exposed state, considered to represent the index tumor, and the biopsy with largest tumor focus in the androgen deprived group.

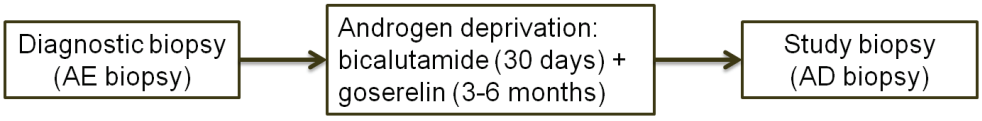

Figure 18: Flow chart of the ADT protocol. Following diagnostic biopsies at the referring hospitals, in Paper II referred to as AE biopsy, patients were subjected to androgen deprivation. Three to six months later, study biopsies, in Paper II referred to as AD biopsy, were taken.

Disadvantages of the approach in Paper II compared to the prostatectomy cohort in Paper I, where whole mount sections were available, include questionable representativeness of biopsies. Expression of proteins may be heterogeneous, and analysis on a small needle biopsy without knowledge of the surrounding tissue, could be insufficient to give a general view of tumor protein expression. On the other hand, the core biopsy is the available material prior to prostate cancer treatment. Thus, if the goal is protein markers for aggressiveness upfront of treatment, analysis of such biopsies in study protocols is desirable. The use of formalin fixed paraffin embedded material from the diagnostic biopsies made analysis of gene expression more difficult. This hampered the analyses in Paper II, where investigation of HIF1-dependent gene expression in prostate cancer patients would have been of interest.

4.2 The CWR22 prostate cancer xenograft model system

In Paper II we generated an experimental *in vivo* model for the effect of ADT on prostate cancer by implanting tissue fragments of the androgen-sensitive CWR22 prostate cancer xenograft subcutaneously in the flank of male Balb/c nude mice aged 6-8 weeks. To reduce differences in the testosterone concentration among the animals, a 12.5 mg sustained-release testosterone pellet was inserted subcutaneously in the neck region. CWR22 was originally harvested from the primary tumor of a patient with high-grade and high-stage prostate cancer (Gleason score 9, M+ disease) (142). The model is known to express PSA and be sensitive to ADT (143). In a previous study, this xenograft model had shown no statistically significant differences in hypoxic fraction between androgen exposed and androgen deprived xenografts at a diameter of 8 mm (144). Thus, to study hypoxia independent HIF1 signaling, this model seemed suitable.

ADT was performed by surgical castration at fixed tumor volumes, and the testosterone pellet was concurrently removed. We analyzed concentration levels of the androgen dependent protein PSA to ensure that castration was efficiently performed. The androgen exposed and androgen deprived PSA levels were 39 and 10 μg/L, respectively (145). Moreover, in another study on the same model system, where the tumors were allowed to regress further before sacrifice, PSA values approaching zero were demonstrated (146), showing a similar PSA pattern as prostate cancer patients on ADT. By comparing hypoxic fractions, transcriptional changes and HIF1α expression at identical volumes in androgen exposed and androgen deprived xenografts, we counteracted the potential effects of tumor volume on gene and protein expression.

4.3 Prostate cancer cell line models

In vitro cell cultures or cell lines are often used as models for cancer disease, due to characteristics such as immortality and rapid replication, which ensure easy access to material. In addition, they provide a reproducible and homogeneous model system. Disadvantages include lack of stromal interaction, unphysiological growth conditions and deviations from their parental disease/cellular system due to long-term passage on plastic.

Four prostate cancer derived cell lines were used in the thesis: 22Rv1 (147), LNCaP (148), DU 145 (149) and PC-3 (150). The cell lines were identity confirmed by short tandem repeat profiling, using Powerplex 16 (151). The only cell line of primary origin is 22Rv1, which is derived from the prostate cancer xenograft CWR22-R, i.e. CWR22 recurring after

castration. 22Rv1 is reported to be weakly stimulated by androgens (147), and is as such androgen responsive. LNCaP is derived from cancer cells metastasized to a lymph node in a patient undergoing ADT for prostate cancer. Both 22Rv1 and LNCaP contain functional AR (152). However, the receptor is truncated in LNCaP, which makes the cells more susceptible to unspecific stimulation through different ligands. LNCaP is also known to turn into androgen independence after a series of passages, and for this reason only passages below 40 were used in our studies (153). DU 145 and PC-3 are cell lines derived from brain and bone metastases, respectively, and are considered to show no expression of AR (152). The cell lines therefore seemed to be appropriate *in vitro* model systems for androgen responsive (22Rv1, LNCaP; Papers I, II) and non-responsive (DU 145, PC-3; Papers I, III) tumors.

4.3.1 Model for tumor hypoxia

A model for hypoxia was created by the use of a humidified hypoxia chamber with integrated control of O_2, CO_2 and N_2 concentrations. Cell lines were plated and allowed to fasten before hypoxia exposure. As a model for chronic hypoxia we exposed the cells to 0.2 % oxygen for 24 hours. From our own laboratory, we had *in vitro* evidence of relevant hypoxia signaling with induction of HIF1α, as well as hypoxia induced radioresistance at this oxygen level. The level is also relevant for the study of hypoxia induced genetic instability (66) and has been found *in vivo* by the use of Eppendorf needles (154). Thus, the model seemed relevant in exploring both hypoxia induced radioresistance and disease aggressiveness.

In Paper I hypoxia inducible genes were assessed by treating the four prostate cancer cell lines with 0.2 % oxygen for 24 hours. Genes that showed a two-fold change in expression as compared to control cells in at least two of four cell lines were identified. This provided us with a comprehensive list of hypoxia responsive genes that could be compared to gene expression in prostate cancer in patients. We included all four cell lines to cover representation of different cancer biologies. In Paper II we exposed the androgen responsive cell lines 22Rv1 and LNCaP to hypoxia to support the prostate cancer relevance of HIF1 targets that were previously derived from chromatin immunoprecipitation studies on breast and hepatocellulary carcinoma cell lines (155, 156). Since we analyzed gene expression changes by ADT in a xenograft system with functional AR, we included only the two cell lines which showed androgen responsiveness. In Paper III we treated the prostate cancer cell lines 22Rv1, PC-3 and DU 145 with 0.2 % hypoxia for 24 hours prior to irradiation to investigate hypoxia induced radioresistance. We included these cell lines to cover a range of vorinostat- and radiation sensitivity. To avoid reoxygenation prior to radiation, closed caps on

the cell flasks were used when we transported the flasks from the hypoxia chamber to the radiation machine located in the adjacent room.

To disclose possible errors, the hypoxia chamber log file containing oxygen detector readings was checked following all experiments. However, we did not include oxygen concentration measurements from the cell cultures exposed to oxygen, but we assumed that it reached a steady state after 24 hours. Reassuringly, radioresistance was observed in the hypoxia-treated cell lines, in addition to expected changes in gene expression as reported in the literature.

4.3.2 Methods for evaluation of radiation response

Following radiation, cells may repair the damaged DNA and progress through S and M phases successfully, as described above in section 2.1.1. Alternatively, they may induce the apoptotic program and undergo controlled cell death. However, immediate apoptosis is probably not the main mechanism for cell death in solid tumors following radiation (157). Often, unsuccessful repair and so-called mitotic catastrophe, where daughter cells inherit DNA with alterations incompatible with life, dominate the response. Secondarily, apoptosis or necrosis, i.e. uncoordinated cell death, may occur, or cell senescence, i.e. permanent conversion to a non-proliferative state (30).

The clonogenic assay examines the ability of single cells to form colonies of fifty cells or more in a tissue culture, termed the clonogenic capacity, and it takes all forms of cell death into account. The assay can be performed on cancer cell lines to evaluate efficacy of different forms of treatments (157). The clonogenic capacity of treated cells and control cells is compared, with the ratio between them, the surviving fraction, taken as a measure of treatment efficacy (30). To ensure that clones contained fifty cells or more, microscopy of candidate clones was performed both in control and treated samples. To avoid bias, a colleague unrelated to the study counted several cell plates blinded to treatment groups (Figure 19). In general, she gained a lower number of clones, but the surviving fractions remained the same, and in accordance with the conclusions of the paper.

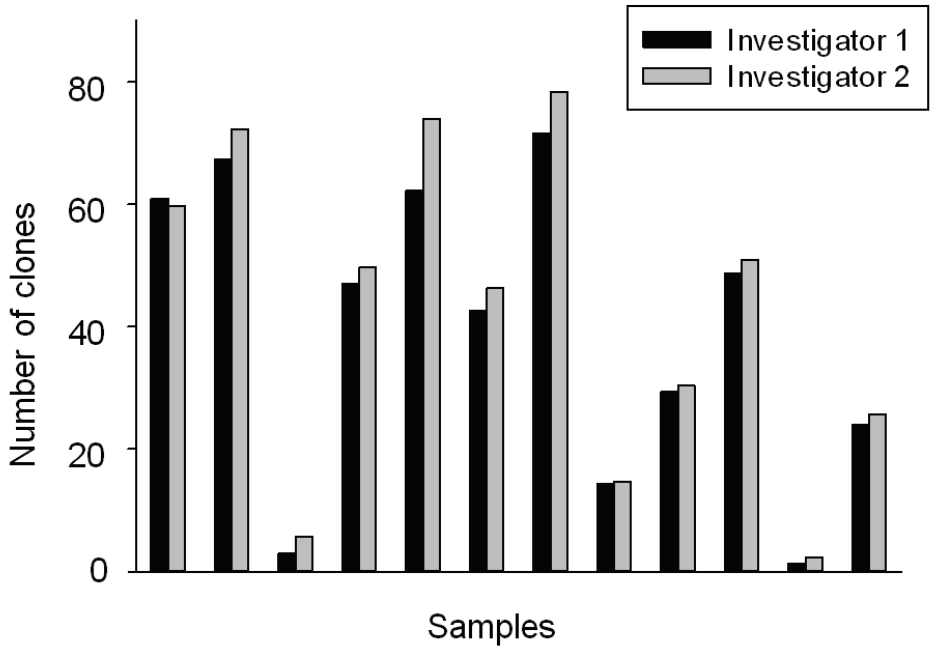

Figure 19: Number of colonies counted by two different investigators, where one was blinded to treatment class.

In the 3D clonogenic assay a different approach in guiding the cut-off for colonies had to be applied, since the cells were lumped together in a sphere. Thus, as an assumption of sphere diameter corresponding to fifty cells, we stained the nuclei with either SYBR Gold or Hoechst, and counted number of nuclei in stacked microscopy immunofluorescence pictures. The sphere diameter corresponding to at least fifty cells was regarded as being the limit for a colony.

4.4 Detection of tumor hypoxia

There are several methods for detecting hypoxia in a tissue, including direct measurement of pO_2 using Eppendorf electrodes. This has been undertaken in several tumor types, including prostate cancer, and remains the gold standard for hypoxia detection (154, 158). Disadvantages include the inability of measuring pO_2 in tumors not accessible from the surface, its invasiveness and the lack of comparison between morphology and oxygen measurements. As a consequence, the pO_2 may by a mistake be read in non-cancerous tissue,

potentially diluting relevant measurements. This is especially important in cancer forms where normal tissue is interspersed in the tumor, as in prostate cancer.

Endogenous and exogenous tissue markers have been used for hypoxia quantification by immunohistochemical approaches. In contrast to pO_2 measurements, use of immunohistochemistry allows direct comparison of hypoxia markers and morphology. Endogenous markers are typically proteins where the expression is induced by hypoxia, for instance HIF1α, solute carrier family 2, member 1 (SLC2A1, alias GLUT1) and carbonic anhydrase 9 (CA9). Disadvantages using endogenous markers include influence of factors other than hypoxia that can affect protein expression levels. For instance can HIF1α levels be dependent on AR activity (Paper II, (78)) and CA9 levels dependent on microenvironment acidity (159), independent of hypoxia.

These limitations are avoided by the use of exogenous markers, where the antigen detected by immunohistochemistry is given to the patient or research animal prior to sampling of material. In both Paper I and II, we used the 2-nitroimidazole pimonidazole as hypoxia biomarker. Pimonidazole, as other 2-nitroimidazoles, forms adducts with amino acids, peptides and proteins in hypoxic cells, with oxygen levels for binding are shown to be below 10 mmHg or 1.3 % oxygen (Figure 20, (160)). Pimonidazole has been shown to mark oxygen gradients independent of relevant enzyme activity (161). Compared to other 2-nitroimidazoles, pimonidazole is of limited toxicity, as doses required for hypoxia detection are low. Disadvantages include the possibility that binding of 2-nitroimidazoles may happen irrespective of hypoxia, as demonstrated in a study of head-and-neck squamous cell carcinoma, where pimonidazole staining under oxic conditions was demonstrated in areas of keratinization (162). It should also be noted that no significant relationship between pimonidazole staining and hypoxia as measured by Eppendorf needles was found in head-and-neck squamous cell carcinoma (137), questioning the comparability of results achieved with the two methods.

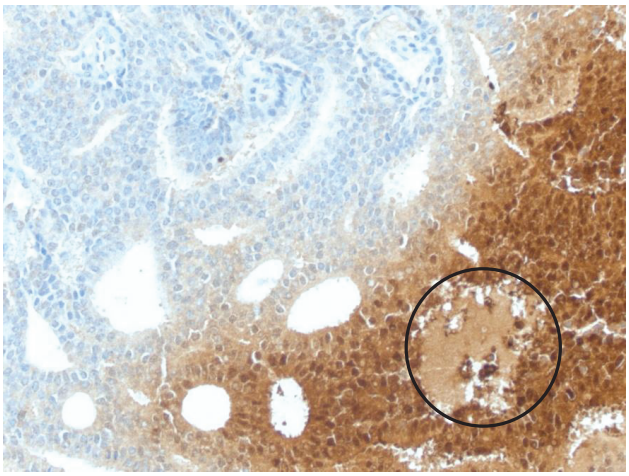

Figure 20: Pimonidazole staining gradient in a prostate cancer section with necrotic area (encircled).

4.5 Gene expression analysis

In this thesis we have studied gene expression in prostate cancer tumors, xenografts and cell lines by whole-genome Illumina HT12 v4.0 gene expression bead arrays containing approximately 47,300 probes. The approach gives an overview of the transcriptome, i.e. the complete set of mRNA transcripts, at a specific time point. Hence, rather than studying single genes, we explore expression of all genes at the same time. Whole-genome analysis makes unsupervised approaches possible, where computer programs can, in an unbiased fashion, analyze differential gene expression between tumors and after specific interventions of cell lines, or identify gene expressions related to biological and clinical information. In particular, transcriptional alterations in all genes involved in the same biological process or signaling pathway can be found, thereby providing strong evidences for a change in tumor phenotypes. This was demonstrated by the gene ontology and gene set analyses in our studies, and the further exploration of the HIF1- and AR pathways. However, although gene expression often is an indication of the encoded protein level, this must be confirmed in separate analyses, as discussed below. In addition, neither regulation of translation nor enzyme activity is provided.

The Illumina method provides reliable results, as was demonstrated in Paper II, where we applied quantitative real-time polymerase chain reaction (qRT PCR) as technical validation of the Illumina data. The threshold level for each gene was set automatically by the software in the logarithmic part of the PCR curve, and the gene expression levels were

analyzed with the delta-delta method, using *B2M* as reference gene. The PCR results showed a strong correlation to the Illumina data, although the genes selected for the validation analysis had expressions within a narrow range (Paper II).

A prerequisite for obtaining reliable data is, however, that high quality RNA is utilized. In particular, in analysis of clinical material obtained after surgery, a concern is the instability of the RNA molecule and a possible degradation before the samples are frozen. To investigate whether this could have occurred in our study, we recorded the ischemia time, as described above in section 4.1.1 and in Paper I.

Downstream analysis of gene expression data

When analyzing 47,300 transcripts at the same time, the risk of obtaining false positive results is imminent. As such, we corrected for multiple testing using the Benjamini-Hochberg algorithm provided in the software (163, 164). We used the Linear Models for Microarray data (LIMMA) analysis to identify differentially regulated genes between patient samples (Paper I) and between treated and untreated cell lines (Paper III). In short, LIMMA uses moderated *t*-statistics to find differentially expressed genes in dichotomized microarray datasets (165). The software provides p-values corrected for multiple testing, which we utilized in Paper III. In Paper I a nominal p-value of 0.05 was applied, to achieve enough genes for the further downstream analyses. In this paper, we included validation of the final results, justifying the use of this approach.

To explore the functional meaning of the LIMMA results, several analysis tools were applied. In all three papers, the gene lists were imported into the Database for Annotation, Visualization and Integrated Discovery (DAVID) software (164). The DAVID software offers several analyses based on the gene ontology (GO) term, which is decided for each gene by a consortium working to unify the annotation of different gene products in relation to their known biological process, cellular component and molecular function (166). Significance Analysis of Microarrays for Gene Sets (SAM-GS) (167) is another approach where enrichment of predefined gene sets, reflecting known signaling pathways or biological processes, were explored by comparing the gene expression profiles of pimonidazole positive and negative tumors (Paper I). Finally, to explore to what extent the HIF1 signaling pathway was changed by vorinostat treatment in Paper III, we utilized network analysis where interactions between proteins encoded by the differentially expressed HIF1 target genes and other genes in the gene list were identified and visualized (168). The functional analysis tools are of utmost importance for the interpretation of gene lists. All methods, however, rely on the

current knowledge of gene function, signaling pathways or protein interactions, which is a major limitation of these approaches.

Prostate cancer validation cohorts

In Paper I gene expression data from two prostate cancer cohorts were used to validate results from the investigation cohort. As analysis of gene expression data carries the risk of over-fitting results to the data, and the use of independent validation cohorts is highly valuable. This approach is facilitated through the Gene Expression Omnibus (GEO) database, where research groups deposit gene expression data together with relevant clinical information. We used two gene expression data sets, one from a prostatectomy patient series (GSE32571; (169)) and one from a watchful waiting patient group (GSE16560; (170)) .

A pitfall of this approach may include differences in the gene expression platform, making comparison between the cohorts difficult. In GSE32571 however, the Illumina bead array platform was used, which allowed us to extract identical probes as used in the investigation cohort. In GSE16560, the cDNA-mediated annealing, selection, ligation and extension platform with only 6100 genes was used. Hence, just 19 of 32 genes in the pimonidazole gene signature were present in this data set, and a separate analysis was required to ensure that this subgroup of genes satisfactorily reflected the pimonidazole staining (Paper I). Reassuringly, expression of every gene in our signature showed correlation to pimonidazole. Moreover, unsupervised clustering of the investigation cohort based on the 19 genes produced two clusters where the pimonidazole immunoscore tended to be higher in the cluster with high gene expression. In addition, the score based on these genes was significantly higher for the pimonidazole positive tumors. This showed that patients clustered in a way that still had connection to pimonidazole staining, although only 19 of the genes were used, allowing us to draw conclusions based on the GSE16560 validation cohort.

Another important aspect in using validation cohorts from the GEO database is the availability of supportive clinico-pathological information. Researchers that upload data to the GEO database add a file with information on these aspects, but the detail level in this file may vary. In GSE32571, the endpoint was Gleason score in the prostatectomy specimen above 7a, thus dividing the cohort in two groups based on a well-defined aggressiveness marker. Unfortunately, follow-up data was not available in this cohort. The GSE16560 data set enabled analysis of pimonidazole-associated gene expression in relation to progression-free and overall survival. As the patients were in a watchful waiting regime, no treatment details were needed. The strength of this cohort was the long follow-up time of up to 30 years,

whereas methodological weaknesses include that patients were diagnosed in the pre-PSA era and they had all stage T1a-b tumors, i.e. non-palpable tumors discovered on TUR-P due to lower urinary tract symptoms. This could question the representativeness to patients diagnosed today. However, aiming for a validation of the pimonidazole gene signature in relation to disease aggressiveness, a cohort of patients not receiving definite treatment was ideal.

AR and HIF1 target genes

In this thesis we utilized published lists of HIF1 (Paper I-III) and AR (Paper I, II) target genes to explore signaling through these transcription factors in hypoxic prostate tumors, and following androgen deprivation of prostate cancer xenografts and hypoxia and vorinostat treatments of prostate cancer cell lines (50, 155, 156). The target lists were published in papers using chromatin immunoprecipitation to analyze transcription factor target sites in the genome, combined with whole-genome transcriptional analysis following interventions (171). The AR targets had been found by stimulating the androgen sensitive cell lines VCaP and LNCaP with androgens (50). We included only genes with elements found to bind AR that were either upregulated or downregulated by androgens (50). However, functional binding sites of androgens may differ in various prostate cancer models, and the AR target list may therefore not have been the correct one for our analysis of gene expression changes in CWR22 xenografts following androgen deprivation. On the other hand, the list was generated based on the union of expression profiles from two different androgen sensitive cell lines, increasing the sensitivity.

For identification of direct HIF1 targets, results from two individual chromatin immunoprecipitation analyses were included (155, 156). As for AR, we included genes with an altered expression following hypoxia treatment of cell lines. A weakness of this approach was the use of cell lines derived from other cancer types than prostate cancer to generate the list, which may reduce its relevance since the hypoxia response may be cell-specific (172). To ensure that the targets of interest were of relevance for prostate cancer, we therefore ensured that they were regulated in prostate cancer cell lines exposed to hypoxia (Paper II).

4.6 Protein expression analysis

For protein studies, we performed Western blot analysis of selected proteins and immunohistochemistry of HIF1α and Ki67. In addition, we used immunohistochemistry to assess the binding of pimonidazole in the tumors. Western blot and immunohistochemistry are

two different techniques using antibody binding and detection systems for protein quantification with different strengths and weaknesses.

Western blot was performed by separating whole cell lysates by sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) as a function of molecular size. We used ladder to control the size and gamma-tubulin as loading control. As such, one can read the expression signal in relation to molecular size, disclosing unspecific antibody binding. A weakness regarding Western blot, however, is the inability to distinguish between cancer and normal tissue. For this reason, the technique was applied only on cell lines, whereas immunohistochemistry was used on material from xenografts and patients.

By immunohistochemistry, distribution of a protein between normal and cancerous cells, as well as distribution within different cellular compartments, can be studied. The technique can, however, not distinguish between specific and unspecific binding. As such, the use of negative controls is important. In Paper I, we included negative controls for the pimonidazole antibody using both irrelevant antibodies on a specimen containing pimonidazole adducts, and applying anti-pimonidazole antibody on a patient who received no pimonidazole. In addition, irrelevant antibodies for Ki67 were applied. None of these negative controls had detectable immunostaining. We therefore concluded that the Ki67 and pimonidazole data were reliable.

In Paper II, negative control for the HIF1α antibody showed staining of the cytoplasmic compartment in cancer cells. We therefore interpreted only staining in the nuclear compartment as true positive in the analysis of HIF1α expression. Of importance for the validation of this antibody was also the observation that Western blots showed positive results only at a molecular size corresponding to the known size of the HIF1α protein, suggesting that unspecific binding was a minor problem.

5. Discussion of results

5.1 Pimonidazole as marker for aggressive, hypoxic prostate cancer

Hypoxia, defined by a pimonidazole positive fraction above 10%, was detected in almost half of the tumors included in the Funcprost protocol (Paper I), in line with previous work suggesting that hypoxia is a common finding in prostate cancer (154, 173). The degree of hypoxia differed considerably both between and within the tumors; in some cases the whole tumor showed strong pimonidazole staining, whereas in others no staining was observed. Correspondingly, staining variation was also found in a study by Carnell and co-workers (139). PO_2 measurements by electrodes, traditionally considered the gold standard of hypoxia detection (38), have also shown heterogeneity in the oxygen level across prostate tumors (154, 173). This large difference in hypoxic fraction between the tumors increases the chance of finding biological and clinical features associated with the hypoxic phenotype, and eases a potential use in the clinic.

We observed positive association between pimonidazole immunoscore and both presence of clinical T3 tumors and lymph node metastasis at diagnosis. This relationship to aggressive features is in line with the study by Carnell and co-workers, where an association between increased pimonidazole staining and high Gleason score was observed (139). The correlating endpoints differed between the studies (Paper I and (139)), possibly due to the limited number of included patients, 39 and 37 respectively, leading to low statistical power. However, presence of clinical T3 stage, lymph node metastasis and high Gleason score are all well validated aggressiveness markers, with high impact on prostate cancer patient outcome (100, 123). All together, these results support a relationship between increased pimonidazole staining and cancer aggressiveness and encourage accomplishment of larger studies to clarify the role of pimonidazole as hypoxia marker in clinical decision making.

5.2 Transcriptional program reflected by pimonidazole staining

To achieve a better understanding of the molecular phenotype reflected by pimonidazole staining, a whole genome transcriptional analysis was performed (Paper I). Pimonidazole positive prostate cancers had enrichment of the biological processes cell cycle, translation and cellular response to stress, and gene lists encompassing proliferation, DNA repair and hypoxia were the most significantly enriched in the following gene set analysis (Paper I). Hypoxia-induced changes in gene expression vary among different cell types (174). Correspondingly,

the hypoxia responsive genes in the transcriptional program showed greatest similarity with the hypoxia gene sets derived from prostate cancer cell lines, whereas those derived from other cancer types were less significant in the gene set analysis (Paper I, (175-180)). An explanation might be that AR regulates many of the same pathways as are activated under hypoxia (50). This was demonstrated in Paper II, where genes in classical hypoxia response pathways like glycolysis showed massive downregulation following androgen deprivation despite comparable hypoxic fractions. Possibly, androgen dependent regulation of such pathways in prostate cancer outweighs the effect from a hypoxic tumor environment and decreases the transcriptional differences between hypoxic and less hypoxic tumors. Hence, no enrichment of the biological processes related to glycolysis was seen in pimonidazole positive tumors in our work (Paper I), in contrast to the findings following androgen deprivation (Paper II). It is also important to keep the oxygen level for pimonidazole staining in mind, which is higher than both radiobiologically relevant hypoxia and the limit used in many studies to identify hypoxia gene sets (58, 181). Thus, this could explain why the transcriptional program associated with pimonidazole staining differ from others identified through prediction analysis of outcome after radiotherapy (175).

The transcriptional program of pimonidazole positive tumors seemed to represent a condition where cells were both hypoxic and proliferative (Paper I). As such, oxygen levels reflected by pimonidazole staining were probably sufficiently high for cell proliferation to occur. Proliferation and hypoxia-related tissue remodeling phenotypes have previously been studied with regard to prostate cancer aggressiveness by Markert and co-workers, where a set of curated gene expression profiles from several cancer forms were analyzed, including the same data set used for validation in our work (Paper I, (16, 170)). In line with our results, combined proliferation and hypoxia-related remodeling gene sets showed a stronger prognostic value in prostate cancer than the hypoxia-related remodeling or proliferation gene sets alone. This was also the case for cancers of the brain and ovary, pointing to generality of the findings (16). It therefore seems that a phenotype comprising both proliferation and hypoxia is particular aggressive not only for prostate cancer, but for several cancer types. Moreover, our work suggests that this phenotype is reflected by pimonidazole staining in prostate cancer.

5.3 Role of HIF1 in the transcriptional program and cancer aggressiveness

Although HIF1 is often upregulated in aggressive cancers (80), we observed no statistically significant associations between HIF1α expression in diagnostic biopsies from the ADT

cohort and Gleason score, clinical T stage, lymph node metastasis or other clinicopathological parameters (Paper II). However, the low number of patients reduced the power of our analysis, which may explain this lack of associations. HIF1α expression has been investigated by several groups in both surgery and radiotherapy cohorts, albeit with differing results (80, 182). In particular, the observation by Weber et al. of *decreased* biochemical failure rate following radiotherapy for prostate cancers with high HIF1α expression is unexpected (182), and points to a more complex regulation of the protein rather than reflection of tumor oxygen levels. Hence, underlying biology reflected by differential HIF1α expression in prostate cancer is not clear (82). Our work shows that androgen deprivation (Paper II) and HDAC inhibition (Paper III) can cause HIF1α downregulation irrespective of hypoxic fraction. HIF1α could reflect other mechanisms important for disease aggressiveness, for instance AR or mTOR activity (78). These observations adds up to the increasing data on hypoxia-independent regulatory mechanisms of HIF1 activity in general (183). HIF1α is therefore probably not a good biomarker of hypoxia in prostate cancer.

HIF1 could, though, play a role in the transcriptional program activated in aggressive, hypoxic prostate tumors. In the pimonidazole gene signature, nine of thirty-two genes were known HIF1 targets (Paper I). This high fraction indicates that genes under regulatory control by HIF1 may have a role in adaptation to hypoxia in prostate cancer. The genes included *FOXM1* and *BIRC5,* which are involved in apoptosis suppression (184, 185). Thus, tumors with high pimonidazole gene score could have activation of anti-apoptotic signaling controlled by HIF1. Moreover, expression of the two genes showed correlation to Ki67 staining (Paper I), in line with the role of HIF1 in cell survival and proliferation. Even though metabolism as biological process was not significantly enriched in the pimonidazole positive tumors, the HIF1 target *PKM*, which is involved in metabolism, was part of the pimonidazole gene score. This points to altered energy metabolism in pimonidazole positive tumors, in line with bona fide biological alterations in hypoxia (37).

5.4 HIF1 signaling and response to ADT and vorinostat

By pimonidazole staining, we had previously demonstrated that the hypoxic fraction was comparable between androgen exposed and androgen deprived state in the CWR22 xenograft at 8 mm diameter (144). This provided us with the opportunity to study possible hypoxia-independent changes in HIF1 signaling following androgen deprivation. Increased oxygenation of prostate cancer following neo-adjuvant ADT prior to radiation therapy could

have clinical implications and had been a matter of debate in the literature (186-188). ADT led to reduced expression of key regulatory enzymes in glycolysis (Paper II), in agreement with the study by Massie and co-workers, where AR activity was shown to have a pivotal role in energy metabolism (50). Moreover, we found that key glycolytic enzymes, such as HK2, PFKFB3, and PKM2 were targets of both AR and HIF1 (50, 155, 156). This may imply that part of the ADT effect on energy metabolism is through reduced HIF1 signaling and occurs independently of changes in the hypoxic fraction of the tumor.

In Paper III, the DU 145 cell line showed downregulation of HIF1α expression and changes in HIF1 dependent signaling following vorinostat treatment under both normoxia and hypoxia. Simultaneously, these alterations were not seen in PC-3, where radiosensitizing effects of vorinostat were minimal. This points to possible involvements of HIF1 signaling in the vorinostat effect related to radiosensitization. The differential radiosensitizing effect of vorinostat on the cell lines was a result of differences in the genetic and epigenetic background between DU 145 and PC-3. Other research groups have reported that DU 145 is more sensitive than PC-3 to vorinostat as single modality (189, 190), but our findings showed that the effect by vorinostat on clonogenic survival was comparable between the cell lines (Paper III). In addition to different assay endpoints, the diverging results between the studies may be due to differences in treatment dose and duration of vorinostat exposure, as these factors are known to affect vorinostat sensitivity (190).

In general, HDAC inhibitors are known to cause HIF1α downregulation, including both the baseline and hypoxia-inducible values. The HDAC inhibitor PCI-24781 was shown to modulate HIF1α expression in lymphoma cell lines, where incubation for 24 hours led to downregulation of HIF1α and increased apoptosis (191). The HDAC inhibitor TSA reduced basal and hypoxia-induced HIF1α protein levels in human tongue squamous cell carcinoma cells *in vitro* without affecting the mRNA levels (192). Vorinostat has been shown to reduce HIF1α expression in lung carcinoma cell lines (193), and in macrophages following hypoxic stress (194). Interestingly, Chong and co-workers noticed an increase in the PHD2 enzyme following vorinostat treatment of macrophages, pointing to a possible post-translational downregulation of HIF1α (194). As discussed, HIF1 is known to be important for survival, proliferation and metastasis of cancer cells in general, and for hypoxic cancer cells in particular (37). Hence, one could speculate that the downregulation of HIF1α in DU 145 phenotypically weakened the cells to such a degree that a second blow by radiation caused the apparent radiosensitizing effect. Correspondingly, since vorinostat at 1 μM concentration did not cause downregulation of HIF1α in PC-3, a radiosensitizing effect of similar magnitude

was not observed. Another possible mechanism is through direct function of HIF1α in the DNA damage response. Interestingly, *DNA-PKcs*, *XRCC5* and *XRCC6* (*KU 80* and *KU 70*, respectively) in NHEJ have been shown to be under transcriptional control by HIF1, and HIF1α can also modify ATM activation following DSBs (195).

5.5 Hypoxia-directed strategies for improved prostate cancer patient care

In this thesis we observed that patients with hypoxic tumors, as measured by the pimonidazole gene score, had aggressive disease with reduced survival in a watchful waiting cohort (Paper I). Other research groups have shown that patients with low prostate cancer pO_2 have significant risk of relapse, metastasis and death despite curatively intended radiotherapy (4, 5). In intermediate and high-risk disease, it has been shown that the combination of radiotherapy and ADT increase the disease-specific and overall survival compared to radiotherapy alone (196-198). In line with this, our gene signature may potentially aid in selecting patients at increased risk of relapse, where intensified treatment is needed. Interestingly, Milosevic and co-workers demonstrated that ADT improves the oxygenation of prostate tissue and related this to the increased radiotherapy efficacy (187). As such, neo-adjuvant ADT could be a possible treatment strategy for hypoxic prostate tumors in general, prior to radiation. For timing of radiotherapy with regard to reoxygenation, it would be useful to have a biomarker for tissue hypoxia. However, we demonstrated that HIF1α is probably not a good hypoxia biomarker during ADT (Paper II).

Other possibilities following identification of hypoxic tumors would be to introduce hypoxia-targeted treatment, as has been successfully done in head and neck cancer by utilization of the drug nimorazole (178). Another method for specific targeting is introduction of so-called contextual synthetic lethality, where the hypoxic tumor microenvironment or activated pathways in hypoxic tumors is targeted (66). In this thesis, we demonstrate that vorinostat decreased the surviving fraction at 2 Gy and 5 Gy under both normoxia and hypoxia in DU 145 (Paper III). In addition, statistically significant radiosensitizing effects were seen in 22Rv1. As such, vorinostat could be a promising drug in high-risk prostate cancer in general, and in hypoxic prostate cancer especially. However, the drug has been shown to induce side effects, and other HDAC inhibitors may provide better improvements (199).

Widespread PSA testing has had the positive effect of identifying high-risk patients at a stage where they are still candidates for curative treatment. However, the other side of the

coin is the identification of patients with low-risk disease who would never experience symptoms from prostate cancer (200). If all these patients were treated with definite treatment forms, unnecessary side effects would be the result. Appreciating this problem, active surveillance has become a management strategy, after introduction by Choo and co-workers in 2002 (200, 201). Enrollment of patients with low-risk disease to active surveillance has been performed in cohort studies, with low rate of progression and cancer-specific death (100, 202). One challenge could be to include other patient groups to active surveillance protocols, for instance a subgroup of patients with Gleason score 7a. In this effort, additional markers to distinguish between lethal and indolent cancer is needed. Interestingly, our gene signature gives independent prognostic information in a watchful waiting series (Paper I). However, a potential value of prognostic information should be validated in a relevant patient group from the post-PSA era.

6. Conclusions and future perspectives

"The challenge to physicians today is to identify the minority of men with aggressive, localized prostate cancer with a natural history that can be altered by definitive local therapy, while sparing the remainder the morbidity of unnecessary treatment"

Zelefsky, Eastham, Sartor and Kantoff;
Chapter 40.6 in Cancer
Principles and Practice of Oncology, 2008,
edited by DeVita, Lawrence and Rosenberg.

Improved patient selection for definitive treatment is highly warranted in prostate cancer. Correspondingly, patients identified as having high risk of relapse are candidates for new, intensified treatment forms. In prostate cancer, hypoxia is related to disease aggressiveness and especially to relapse following radiotherapy. As such, hypoxia is both a potential prognostic biomarker and a target for intervention. Thus, the overall aim of this thesis was to investigate whole-genome transcriptional programs in prostate cancer related to hypoxia and treatment interventions.

To conclude, we have presented a gene signature characteristic of hypoxic prostate cancer. Expression level of the genes showed independent, prognostic value when corrected for known markers of disease aggressiveness in a patient cohort not receiving treatment. This is promising, and motivates for validation studies in patient cohorts from the post-PSA era subjected to active surveillance, surgery or radiotherapy. In addition, increased insight into the molecular background of aggressive, hypoxic tumors was achieved that could reveal key pathways in aggressive disease, thus opening up for directed treatment of hypoxic prostate cancer. Hence, we are currently recruiting more patients in the Funcprost protocol and follow-up data are collected. In addition, the protocol includes evaluation of other hypoxia markers, including multiparametrical MRI, which allows repeat, non-invasive measurements to be taken.

Patients identified at high risk of relapse by established prognostic markers, and perhaps especially patients with hypoxic tumors, should receive improved treatment. This could be performed by refining current treatment strategies. One possibility would be to adjust radiotherapy initiation to the point where neo-adjuvant ADT had improved tumor oxygenation. In this thesis we provide proof-of-principle evidence that expression of HIF1α is

not a good marker for the changes in hypoxic fraction by ADT, since HIF1α expression and HIF1 dependent gene transcription were found to be affected by ADT independently of hypoxia. A more compelling strategy could be to introduce imaging parameters to evaluate tissue hypoxia during a course of neo-adjuvant ADT.

Another way of treating hypoxic, aggressive tumors would be to *intensify* the treatment. We provide pre-clinical evidence that the HDAC inhibitor vorinostat has radiosensitizing effects, with sustained effect under experimental, hypoxic conditions. Thus, radiosensitization by HDAC inhibitors is a possible strategy to overcome treatment failure due to hypoxic tumors. However, the effect was variable between cell lines, and further experimental *in vivo* validation should include search for biomarkers.

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