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Mechanisms Behind Growth Of Castration-Resistant Prostate Cancer Bone Metastases

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To my parents

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ABSTRACT

Background: The first-line treatment for patients with advanced prostate cancer (PC) is androgen deprivation therapy. This therapy is initially effective, but after some time tumors relapse, predominantly within the bone, and are then termed castration-resistant prostate cancer (CRPC). The majority of CRPC tumors show androgen receptor (AR) activity despite castrate levels of circulating testosterone. AR activity could be caused by several mechanisms including; intratumoral androgen synthesis, *AR* amplification, *AR* mutations and expression of AR splice variants. The mechanisms controlling CRPC growth in the clinically most relevant metastatic site, the bone, are not fully identified. The purpose of this thesis was therefore to explore AR expression and possible mechanisms behind CRPC growth in PC bone metastases in order to find mechanisms that could be targeted for treatment and/or predict response to certain therapies.

Materials and Methods: We have examined hormone-naïve and CRPC bone metastases samples obtained from patients at metastasis surgery, non-malignant and malignant prostate samples obtained from patients at radical prostatectomy, and PC cell lines cultured *in vitro*. Analysis has been performed using RT-PCR, whole-genome expression arrays, immunohistochemistry, western blotting, FISH, copy number assays and gene ontology analysis. Functional studies have been made by protein overexpression and knock-down in PC cells *in vitro* and effects studied by evaluation of cell viability, migration, and invasion.

Results: We found that high nuclear AR immunostaining (presumed to reflect high AR activity) in bone metastases from CRPC patients was associated with a particularly poor prognosis, while no difference in AR staining was observed between hormone-naïve and CRPC metastases. Further, expression of AR splice variants (*AR-V7, AR-V567es*) was associated with a high nuclear AR immunostaining score and shown to be increased in CRPC compared to hormone-naïve bone metastases. High levels (levels in the upper quartile) of AR splice variants in CRPC bone metastases was related to disturbed cell cycle regulation and short patients survival. No differences in steroidogenic enzyme levels were detected between CRPC and hormone-naïve bone metastases. Higher levels of enzymes involved in late steps of androgen synthesis (adrenal gland steroid conversion) were observed in bone metastases than in non-malignant and/or malignant prostate tissue, while the enzyme levels in earlier steps (*de novo* steroidogenesis) were lower in bone metastases. A subgroup of metastases expressed very high levels of AKR1C3, indicating that this group may have an induced capacity of converting adrenal-gland derived steroids into more potent androgens. This was not associated to CRPC but merely with the advanced stage of metastasis. High protein levels of AR splice variants were found in bone metastases with low AKR1C3 levels, while metastases with high AKR1C3 levels primarily contained low AR variant levels. Furthermore, about half of the CRPC bone metastases showed *AR* amplification which was associated with co-amplification of *YIPF6*, and a gene expression pattern that pointed at decreased osteoclast activity, and consequently decreased bone resorption.

Conclusions: The majority of CRPC bone metastases show high nuclear AR immunostaining that seems to be associated with a particularly unfavorable outcome after metastasis surgery. Subgroups of CRPC bone metastases could be identified according to presence of *AR* amplification and expression levels of AKR1C3 or AR splice variants, which might have clinical relevance for treatment of PC patients.

ORIGINAL PAPERS

The thesis is based on the following papers, which are referred to in the text by their roman numerals:

PAPER I

Nuclear androgen receptor staining in bone metastases is related to a poor outcome in prostate cancer patients.

Crnalic S, Hörnberg E, Wikström P, Lerner U H, Tieva Å, Svensson O, Widmark A and Bergh A. Endocrine-Related Cancer 2010;17:885-895.

PAPER II

Expression of androgen receptor splice variants in prostate cancer bone metastases is associated with castration-resistance and short survival.

Hörnberg E, Ylitalo B E, Crnalic S, Antti H, Stattin P, Widmark A, Bergh A and Wikström P. PLoS ONE 2011; 6(4):e19059.

PAPER III

Characterization of prostate cancer bone metastases according to expression levels of steroidogenic enzymes and androgen receptor splice variants.

Jernberg E, Thysell E, Ylitalo B E, Rudolfsson S, Crnalic S, Widmark A, Bergh A and Wikström P. PLoS ONE 2013; 8(11):e77407.

PAPER IV

Molecular features of prostate cancer bone metastases harboring androgen receptor gene amplification.

Jernberg E, Brattsand M, Thysell E, Golovleva I, Lundberg P, Crnalic S, Antti H, Widmark A, Bergh A, Wikström P.

Manuscript

ABBREVIATIONS

ADT	Androgen deprivation therapy
AKR1C3	Aldo-keto reductase family 1, member C3
AR	Androgen receptor
ARE	Androgen-responsive elements
ARfl	Androgen receptor full length
AR-Vs	Androgen receptor splice variants
BPH	Benign prostate hyperplasia
CRPC	Castration-resistant prostate cancer
DHEA	Dihydroepiandrostenedione
DHT	Dihydrotestosterone
GS	Gleason score
HN	Hormone-naïve (untreated)
IHC	Immunohistochemistry
LBD	Ligand-binding domain
MB	Mega base pairs (1 million base pairs)
NE	Neuroendocrine
PC	Prostate cancer
PSA	Prostate specific antigen
RT-PCR	Reverse transcriptase polymerase chain reaction

POPULÄRVETENSKAPLIG SAMMANFATTNING

Bakgrund

Prostatacancer är den vanligaste maligna tumörformen hos svenska män. Varje år diagnostiseras närmare 10 000 män med prostatacancer och ca en fjärdedel av dessa kommer att dö av sjukdomen. När prostatatumörerna är små och lokaliserade till prostatan kan de botas genom operation eller strålbehandling. Om tumören däremot har spridit sig med dottertumörer till andra organ finns ingen botande behandling. I dessa fall behandlas patienten med kastrationsbehandling som syftar till att sänka nivåerna av manligt könshormon, eftersom könshormon stimulerar tumörtillväxten. Kastrationsbehandling bromsar tumörtillväxten och lindrar sjukdomen en tid men förr eller senare återkommer tumören. När tumören börjar växa igen trots kastrationsbehandling kallas den kastrationsresistent, vilket betyder att även fast nivåerna av manligt könshormon är låga kan tumören med hjälp av olika mekanismer växa ändå. Prostatacancertumörer kan ge upphov till metastaser (dottertumörer) i bland annat lunga, lever och lymfkörtlar men det i särklass vanligaste stället är i skelettet. Skelettmetastaser orsakar stort lidande för patienten i form av smärtor, patologiska frakturer och ryggmärgskompression.

Det finns flertalet kända möjliga mekanismerna för hur prostatatumörer kan växa trots kastrationsbehandling men lite är känt om dessa mekanismer i skelettmetastaser. Syftet men denna avhandling var därför att undersöka några av dessa mekanismer i kastrationsresistenta skelettmetastaser från patienter med prostatacancer med det långsiktiga målet att lära oss mer om hur dessa patienter bäst kan behandlas.

Material och metoder

Vi har analyserat vävnadsprover från skelettmetastaser och från lokala prostatatumörer och även använt cellsystem. Proverna har analyserats med mikroskopiska och molekylärbiologiska metoder.

Resultat

Våra studier visade att de flesta av de kastrationsresistenta skelettmetastaserna hade höga nivåer av androgenreceptorn, som är cellernas målmolekyl för manliga könshormon och den som därmed förmedlar könshormonernas celleffekter. Högt uttryck av denna receptor visade sig vara förenat med sämre överlevnad hos dessa

patienter. I vårt andra arbete studerade vi uttrycket av en typ av androgenreceptor som är strukturellt förändrad vilket gör att den alltid är aktiv utan att något manligt könshormon har bundit till den. Detta skulle kunna betyda att en tumör kan fortsätta växa trots att patienten genomgått kastrationsbehandling. Det visade sig att en subgrupp av patienter med höga nivåer av den konstitutivt aktiva androgenreceptorn hade högt genuttryck av gener kopplade till celldelning och avled tidigare i sin sjukdom än övriga. Vi studerade även en annan mekanism hos kastrationsresistenta tumörer där tumörcellerna själva börjar syntetisera manliga könshormoner. Många enzymatiska steg är involverade i denna process och vi såg att några av dessa hade väldigt höga nivåer i metastaser jämfört med i tumörer som var lokaliserade till prostatan. I vårt sista arbete utvärderade vi hur stor andel av skelettmetastaserna som hade fler genkopior av androgenreceptorn än vad en cell normalt ska ha. Detta är ett sätt för tumören att öka sitt uttryck av androgenreceptorn och på så sätt lättare kunna växa trots kastrationsbehandling. Vi fann att ca hälften av de kastrationsresistenta benmetastaserna hade ökade kopietal av androgenreceptorn och fortsatta studier pågår där vi försöker förstå hur detta påverkar skelettmetastasers växtsätt.

Slutsatser

Sammanfattningsvis visar våra studier att androgenreceptorn har en viktig roll i utvecklingen och tillväxten av skelettmetastaser vid prostatacancer. Metastaserna kan delas in i subgrupper med avseende på de mekanismer som tumören använder för att växa trots kastrationsbehandling. Detta kan ha betydelse för vilken typ av behandling som bör ges till patienter med skelettmetastaser vid prostatacancer.

INTRODUCTION

THE PROSTATE

Prostate function

The prostate gland is located in front of the rectum, just below the urinary bladder, and surrounds the urethra. The main function of the prostate is to synthesize and secrete proteins and fluids that together with contributions from the seminal vesicles form most of the ejaculate. Although the prostate is involved in fertility, it is not required for reproduction. The major protein produced by the prostate is a protease, prostate specific antigen (PSA), that helps to liquefy the semen so that the sperms way to the egg is facilitated [1]. Normally PSA is secreted into the prostate lumen, transported to the urethra and removed during ejaculation. During conditions such as prostate cancer, benign prostate hyperplasia (BPH) and inflammation the basal epithelial layer and basal membrane are disrupted and PSA leaks into the surrounding stroma and vasculature. Thereby PSA can be elevated in the blood and used as a diagnostic marker for prostate diseases [2].

PROSTATE AND TUMOR GROWTH CONTROL

Action of testosterone

The prostate is androgen regulated and dependent on androgens for development, growth and function [3]. Testosterone is the main circulating androgen, and is synthesized by the Leydig cells in the testis. Production of testosterone is stimulated by the hypothalamus through luteinizing hormone releasing hormone (LHRH), which activates the pituitary gland to produce luteinizing hormone (LH), which in turn stimulates the Leydig cells. In addition, there is a negative feedback loop where testosterone inhibits the release of LHRH in order to maintain the circulating testosterone within normal levels. A small proportion (5-10 %) of testosterone is produced by the adrenal glands. In the prostate, testosterone is converted by 5-alpha reductase to the more potent androgen dihydrotestosterone (DHT) [4, 5]. Testosterone and DHT bind to and activate the androgen receptor (AR). Without androgens the AR is located in the cytoplasm and bound to chaperones. Binding of testosterone or DHT induces a conformational change in the receptor structure that leads to dissociation of chaperone proteins. The AR dimerizes and translocates to the nucleus and interacts with transcriptional co-activators and co-repressors, binds to androgen-response elements (ARE) and

regulates gene transcription of AR target genes such as *PSA, TMPRSS2, NKX3.1* and *FKBP5* [6].

Hormonal ablation

In 1966, Charles Huggins was awarded the Nobel Prize for his discovery that castration led to shrinkage of the prostate gland [7, 8]. When the supply of androgens is depleted or when androgen action is blocked, by castration or anti-androgen treatments respectively, the prostate luminal epithelial cells undergo apoptosis [9-11] . However, both stromal and basal epithelial cells are maintained during castration [12, 13]. It is known that the prostate epithelial growth and regression are not regulated only by direct effects of androgens on the luminal epithelial cells but also indirectly through paracrine factors from the stroma and vasculature. AR is expressed in the luminal epithelial and stromal cells but not in basal epithelial cells, neuroendocrine (NE) cells and prostate stem cells. Studies in transplanted tissue recombinants and in animals where the AR is selectively knocked-out in the epithelium or in the stroma, show that castration induced apoptosis is mediated via ARs in the stroma, and not via epithelial ARs. The AR in the mature epithelium is found to maintain differentiation and to suppress proliferation of these cells [14-17]. In prostate cancer cells the paracrine mechanism of androgen-stimulated growth is converted to an autocrine mechanism, where AR signaling in the tumor cells directly activates the production of autocrine growth factors [18].

Androgen receptor structure

The AR is a member of the steroid hormone receptor transcription factor superfamily. The *AR* gene is located on chromosome X (Xq11-12) and contains eight exons encoding a 110 kDa protein (Figure 1). The AR protein consists of a NH_2 terminal transactivation domain (NTD, encoded by exon 1), a DNA binding domain (DBD, encoded by exons 2 and 3), a hinge region (H, encoded by the 5′ portion of exon 4) which contains the nuclear localization signal, and a ligand-binding domain (LBD/CTD, encoded by the remainder of exon 4 through 8) [19].

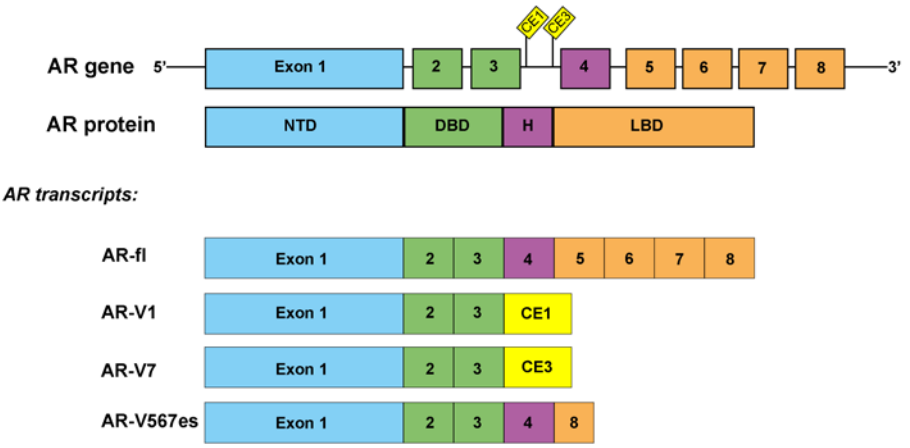

Figure 1. Schematic illustration of the structure of the *AR* full length transcript and protein and a schematic structure of the transcripts for the *AR* splice variants studied in this thesis; *AR-V1*, *AR-V7* and *AR-V567es*. NTD, N terminal domain; DBD, DNA binding domain; H, hinge region; LBD, ligand binding domain; CE, cryptic exon.

PROSTATE CANCER

General background

Prostate cancer is the most common cancer in men in Sweden. In 2011, 9663 new cases of prostate cancer were diagnosed and about 25 % of them will die from their disease (The National Board of Health and Welfare, Sweden). The incidence of prostate cancer has increased over the last 20 years probably caused by the introduction of PSA testing. Prostate cancer is mainly a disease of the elderly and most of the men are diagnosed with prostate cancer between ages of 70-74 years, while the majority of prostate cancer deaths occur in men over 79 years. The single most significant risk factor for developing prostate cancer is advanced age. The incidence and mortality for prostate cancer varies in different regions around the world and the environment and diet/lifestyle could be important factors that may explain those differences. In addition, the genetic background may also effect the disease risk [20, 21].

Diagnosis and prognosis of prostate cancer

Measurement of the PSA level in blood is used to assess the risk of having prostate cancer. A PSA value < 3 ng/ml is considered normal and a PSA value > 10 ng/ml indicates that the patient has a substantial risk of having prostate cancer. The

majority of patients have a PSA value between 3-10 ng/ml at diagnosis, which could be caused by other conditions besides prostate cancer, such as BPH and prostatitis. Ultrasound guided needle biopsies are taken from the prostate in patients with elevated PSA levels, and if a biopsy contains cancer it is scored according to the Gleason system [22]. The differentiation pattern of the tumor is scored on a scale ranging from 1 to 5, where 5 represents the less differentiated and most aggressive tumor pattern. The most common and the second most common area of differentiation are summarized into the Gleason score (GS) (2-10). GS is the strongest prognostic tool available today for prostate cancer. GS is a good predictor of outcome in patients with low GS < 6 or high GS 8-10, but the majority of patients have GS 6-7 where the outcome is very variable and today largely unpredictable [23]. Methods used to determine if the prostate cancer is local (not spread outside the fibrous capsule), locally advanced (spread outside the fibrous capsule but no metastases) or advanced (metastatic disease) are digital rectal exam and bone scintography.

Prostate cancer metastasis

Prostate cancer metastasizes predominantly to the bone. Other sites for metastases are lungs, liver and lymph nodes. According to one autopsy study about 80 % of men with advanced prostate cancer had bone metastases [24]. Metastases to the bone can lead to replacement of the bone marrow, spinal cord compression, severe bone pain, cachexia and death[25, 26]. In patients with localized prostate cancer the 5-year survival rate is almost 100%, while in patients with distant metastases the 5-year survival rate is decreased to 31% [27]. One of the major questions about prostate cancer bone metastases is, "why bone?" There are two possible general explanations to this. The first is the hemodynamic hypothesis proposed by Ewing in 1928 which says that the metastatic dissemination occurs by mechanical factors that are a result of the anatomical structure of the vasculature system. This would mean that blood flow from the prostate preferentially would reach the skeleton, and by that an enhanced delivery of circulating prostate cancer cells to this site. However, when Weiss and Sugarbaker reviewed clinical data on site preferences by different cancers they concluded that regional metastasis could be explained by anatomical and mechanical reasons, but metastasis to distant organs were site specific [28, 29]. The second, and today the most believed explanation is the "seed and soil" hypothesis proposed by Paget in 1889. According to this theory the predilection of certain tumors to spread to certain organs involves the existence of

specific favorable interactions between tumor cells ("the seed") and the microenvironments at the metastatic site ("the soil") [30]. The exact reasons for preferential establishment and growth of prostate cancer cells in the skeleton are today not known. The bone metastases in prostate cancer have primarily an osteoblastic/sclerotic phenotype, thus shifting the balance in the remodeling process to new bone formation [31, 32]. This is in contrast to most other adenocarcinomas that form lytic bone lesions which can be explained by the vicious cycle where tumor cells trigger bone resorption via secretion of parathyroid hormone-related protein (PTHRP) and other osteoclast stimulating factors, that in turns stimulates tumor cells by the release of growth factors such as TGF-β and IGF-1 from the bone matrix [33]. This interaction between tumor cells and the bone microenvironment results in a vicious cycle of bone destruction and tumor growth.

Treatment of local and metastatic disease

Patients with localized prostate cancer can be cured by radical prostatectomy or radiotherapy. If the life expectancy of the patient is long and the tumor is at an early stage it is common that the patient is subjected to active monitoring (treatment when signs of tumor progression are detected), and an early stage tumor in a patient with short life expectancy can instead be subjected to watchful waiting (no treatment until symptoms of metastases). About 25 % of the patients with a localized prostate cancer treated with radical prostatectomy or radiotherapy relapses. For advanced and metastatic prostate cancer there is no cure and the therapy is palliative in the form castration therapy, also called androgen deprivation therapy (ADT), which lowers the androgen levels with either a gonadotropin-releasing hormone analogue or with surgical orchiectomy.

CASTRATION-RESISTANT PROSTATE CANCER

Initially the castration therapy reduces tumor size and pain associated with metastases but unfortunately the majority of tumors relapse within a few years into what is termed castration-resistant PC (CRPC). Despite castrate levels of circulating testosterone (< 50ng/dL) the AR and its transcriptional output most frequently remain expressed in CRPC tumors. Numerous mechanisms have been implicated in the aberrant AR reactivation. Those include *AR* amplification, expression of AR splice variants, intratumoral androgen synthesis, *AR* mutations,

changes in the coregulatory components of the AR complex and finally activation of AR complex via cross-talk with other signaling pathways [34, 35]. These mechanisms are described in more detail below.

Treatment of castration-resistant prostate cancer (Figure 2)
Given that AR signaling remains active in CRPC patients it is recommended that ADT should be continued by adding an AR antagonist such as bicalutamide to the castration therapy (combined androgen blockade). The first-line treatment for patients who relapse after ADT are chemotherapy in form of docetaxel [36, 37]. The second line approved treatment options are cabazitaxel [38] (chemotherapy), abiraterone acetate [39] (CYP17 inhibitor), enzalutamide [40] (AR antagonist/AR inhibitor), and alpharadin [41] (Radium 233 radioisotope).

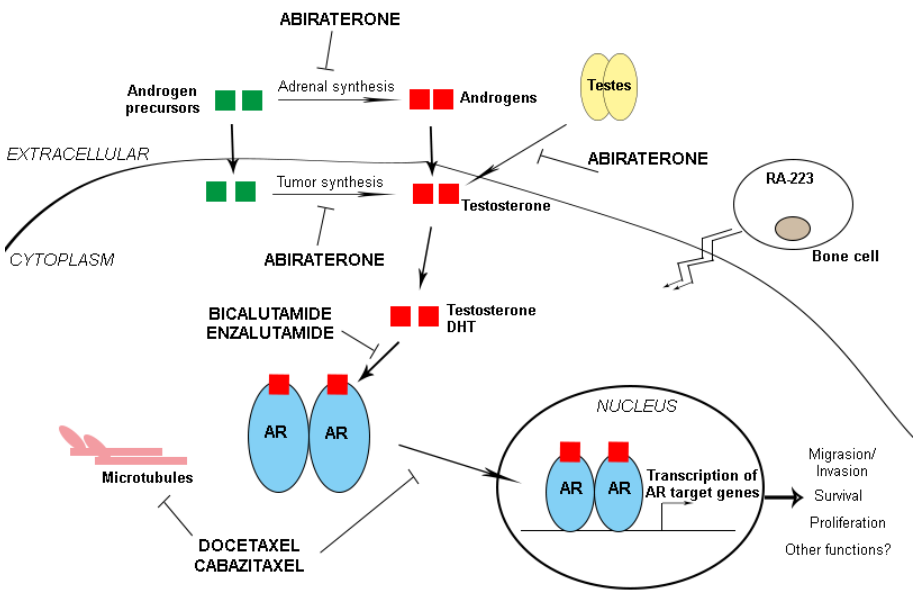

Figure 2. Schematic illustration of the mechanisms of action of drugs used in castration-resistant prostate cancer. Abiraterone is a CYP17 inhibitor that blocks androgen synthesis not only in the testis but also in the adrenal gland and in tumor tissue. Bicalutamide and enzalutamide are AR antagonists that bind the AR ligand site, thus preventing ligands to bind to the AR. Cabazitaxel and docetaxel are taxanes that stabilizes microtubule which results in blocking of cell division, thereby inducing cell death, taxanes also inhibits nuclear translocation of the AR [42]. Alpharadin (Radium 233 radioisotope) targets new bone growth in and around bone metastases and induces double-strand DNA breaks through alpha radiation over a short distance, thereby inducing cell death. (Redrawn and modified according to [35, 43]).

Androgen receptor gene amplification

Gain, or amplification of the *AR* is one of the most frequent genetic alterations in CRPC. Different studies have shown that about 20-30 % of locally recurrent CRPC tumors have *AR* amplification [44-46]. In contrast, tumors from untreated prostate cancer patients very rarely contain *AR* amplification. For example, in a study by Bubendorf *et al* 1999, *AR* amplification was detected in only 2 of 205 of the untreated prostate cancer patients [46], suggesting that amplification is selected for during the emergence of CRPC. *AR* amplification has been shown to induce AR levels and thereby to sensitize the cells to low levels of androgens and restore AR-regulated gene expression [47, 48].

Androgen receptor splice variants

The ligand-binding domain (LBD) of the *AR* is encoded by exons 4-8, and is not essential for transcriptional activity [49]. Androgen receptor splice variants (AR-Vs) lack the LBD and are proposed to be constitutively active (Figure 1).

The first gain of function AR-Vs were identified in 22Rv1 cells due to the presence of a smaller, 75-80 kDa AR immunoreactive species on western blot that was initially thought to be a proteolytic degradation fragment of full length AR [50]. However, later work demonstrated that RNA interference (RNAi) targeted against the LBD of the *AR* reduced expression of *AR* full length but not of the shorter isoforms [51]. These data suggested that the truncated AR-Vs were not a product of full length AR mRNA or protein, but instead derived from unique RNAs.

Today AR-Vs are said to arise as a result of the incorporation of alternative, or cryptic exons, coded for in the *AR* [51-53] or through an exon skipping mechanism in which non-contiguous AR exons are spliced together [54]. Since the identification of AR-Vs nearly a dozen different *AR-V* mRNA have been identified [55]. To date, the AR-Vs named AR-V1, AR-V7 (also named AR3), AR-V9 and AR-V567es are the variants found in human tissue specimens [52-54, 56] and additional AR variants have been detected in human PC cell lines [51, 52, 56-58]. Studies in PC cell lines that naturally express AR variants (22Rv1, CWR-R1 and VCaP) have revealed that the AR variants show androgen-independent effects on growth and expression of AR target genes. Increased levels of AR-Vs have been detected in CRPC tumors when compared to non-treated primary tumor tissue [52-54].

Two of the best characterized AR-Vs are AR-V7 and AR-V567es. AR-V7 was originally discovered and functionally tested in the CRPC 22Rv1 and CWR-R1 cell lines [51-53]. The AR-V7 is coded for by AR exons 1-3 and a terminal 35-kb cryptic exon in intron 3, named CE3 [53]. Overexpression of AR-V7 in LNCaP cells and specific depletion of endogenous AR-Vs in 22Rv1 cells result in increased and decreased growth, respectively, under CRPC-like *in vitro* and *in vivo* conditions [51, 52, 58]. AR-V567es was first identified in LuCaP 86.2 and 136 PC xenografts and are coded for by exons 1-4, and because of a frame-shift due to loss of exons 5-7, exon 8 has a stop codon generated after the first 10 amino acids resulting in a shortened exon 8. AR-V567es increases proliferation of LNCaP cells in the absence of androgen as well as enhanced proliferation in response to very low levels of androgen. When castrated, the mRNA levels of the AR-V567es increased in the LuCaP xenografts compared with intact hosts [54].

Intratumoral androgen synthesis

In recent years, several studies have shown that intraprostatic testosterone and DHT levels do not decline as markedly as serum levels after ADT. Prostate tissue levels of DHT in prostate cancer patients treated with ADT therapy before prostatectomy declined by only ~ 75 % versus ~ 95 % in serum levels [59, 60]. Furthermore, in CRPC tumors intratumoral androgens (testosterone, DHT) are maintained at levels sufficient to activate the AR signaling pathway [61-65]. One hypothesis is that intratumoral androgen synthesis in CRPC tumors provide an adaptive response to ADT, facilitating CRPC tumor survival in a castrate environment with low levels of exogenous androgens. The maintenance of intratumoral androgens can be accounted for by intratumoral synthesis of steroid hormones, either via the uptake and conversion of adrenal androgens into more potent steroids [66] or via *de novo* steroidogenesis from cholesterol [67, 68] (Figure 3). Several studies have identified increased expression of enzymes mediating testosterone and DHT synthesis from weak adrenal androgens. For example; transcript levels of *AKR1C3, SRD5A1* and *HSD3B2* have been reported with an increased expression in CRPC tumors [65, 69, 70]. Studies using LNCaP cells and a LNCaP xenograft model for CRPC identified that enzymes required for *de novo* steroid synthesis, including CYP11A1 and CYP17A1 are expressed and may be increased in castration-resistant sublines [67, 68, 71]. These tumors could also synthesize testosterone and DHT from radio-labeled cholesterol *in vitro* and *in vivo*. Although prostate cancer cell lines and xenografts can synthesize detectable

levels of androgens *de novo*, it is not clear whether this occurs in CRPC patients at levels that are adequate to fully reactive AR. One study found very low levels of *CYP17A1* and *HSD3B1* mRNA in locally recurrent CRPC [70] whereas both these enzymes and CYP11A1 were readily detected in metastatic CRPC [69].

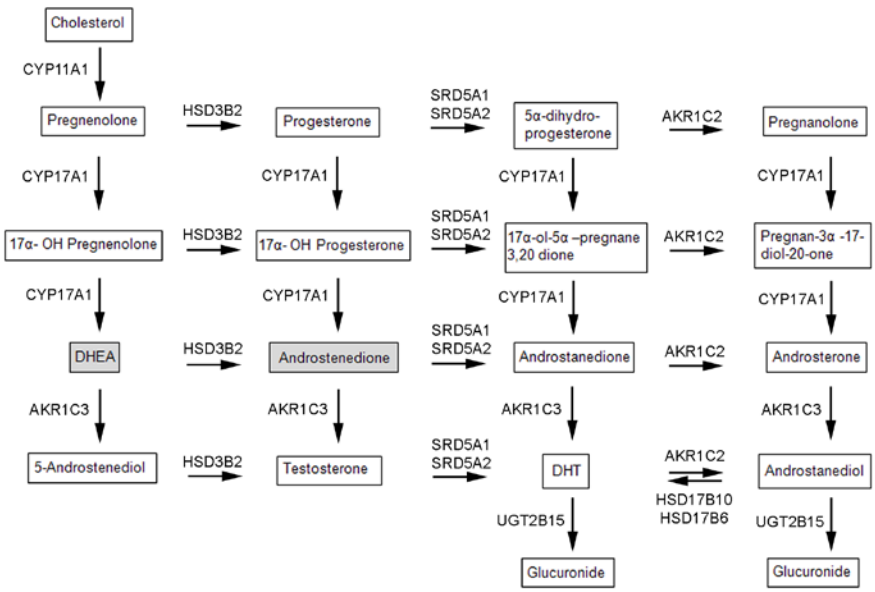

Figure 3. Simplified scheme of the key enzymes involved in testosterone and DHT steroidogenesis. Steroids secreted by the adrenal gland are highlighted in gray.

Below follows a short description of the remaining suggested mechanisms behind castration-resistant prostate cancer. These mechanisms may be as important as the ones described above but have not been studied within this thesis.

Androgen receptor mutations

AR mutations are very rare in early stage untreated prostate cancer but in CRPC they are present in approximately 10-30 % [72-78]. Most of the mutations in the AR are found in the LBD and result in broaden ligand specificity which can lead to AR activation by weak adrenal androgens and other steroid hormones, including dihydroepiandrostenedione (DHEA), progesterone, estrogen and cortisol [72, 79-83]. A subset of these mutations also converts known AR antagonists (flutamide or bicalutamide) into agonists and it has been proposed that treatment with specific AR antagonists selects for tumors expressing AR mutants activated by the

therapeutic agent [84]. The most frequent and first identified point mutation of the AR in prostate cancer is the T877A mutation [85]. It has been found in the LNCaP cell line and moreover in CRPC tissues. The T877A mutation is located in the LBD and alters the stereochemistry of the binding pocket [86]. The mutation results in LNCaP cell growth *in vitro* in response to androgens and also in response to other steroids (estrogen and progesterone) and the AR antagonist hydroxyflutamide [87].

Coregulators of the androgen receptor

AR regulates gene expression through recruitment of a series of coregulator complexes [88, 89]. These coregulators can function either as coactivators enhancing transcription or as corepressors suppressing transcription of AR target genes. It is generally accepted that AR agonists induce the recruitment of coactivators, whereas AR antagonists influence conformational changes that promote the recruitment of corepressors. Cell based studies have shown that dysregulated expression of coregulators can increase overall AR activity particularly at low hormone levels as well as broaden the ligand specificity. Several coactivators, including SRC-1, SRC-2/TIF-2, SRC-3, TIP60 and ARA70 have been reported to be increased in recurrent prostate cancer [90-94].

Interaction of the androgen receptor with other signaling pathways

Activation of several signal transduction pathways in CRPC have been shown to enhance AR activity in an environment where androgen levels are low or even absent. Growth factor receptors such as IGF-1R, IL-6R, and EGFR induce activation of important growth and survival pathways including MAPK, AKT and STAT signaling [35, 95, 96]. Increased expression of HER2/Neu receptor tyrosine kinase is associated with aggressive primary prostate cancer and CRPC [97-100]. HER2 can also facilitate AR activation in response to IL-6, a cytokine with increased levels in the serum of patients with metastatic prostate cancer [101].

Taken together the suggested mechanisms behind CRPC have in general been thoroughly studied in model systems for prostate cancer but their relevance in clinical materials is not so well documented.

AIMS

GENERAL AIM

The bone is the clinically most relevant metastatic site for prostate cancer. The mechanisms regulating growth of prostate cancer bone metastases are not fully understood. Most of our current knowledge about prostate cancer and mechanisms behind treatment failure is based on studies of primary tumors or soft tissue metastases, and not on studies of bone metastases. The general aim of this thesis was therefore to explore possible mechanisms behind castration-resistant prostate cancer (CRPC) growth in prostate cancer bone metastases in order to find mechanisms that could be targeted for treatment and/or predict response to certain therapies.

SPECIFIC AIMS

Paper I

To explore androgen receptor expression and possible down-stream effects of the androgen receptor activity such as cell proliferation, apoptosis and prostate specific antigen expression and their relation to survival in prostate cancer bone metastases

Paper II

To study levels of androgen receptor splice variants in hormone-naïve and CRPC bone metastases in comparison to non-malignant and malignant prostate tissue, as well as in relation to androgen receptor protein expression, transcription profiles and patient survival

Paper III

To examine if CRPC bone metastases express higher levels of steroid-converting enzymes than untreated bone metastases and to analyze steroidogenic enzyme levels in relation to expression of androgen receptor splice variants

Paper IV

To study androgen receptor gene amplification in hormone-naïve and CRPC bone metastases and in addition explore specific molecular and functional consequences of this

MATERIALS AND METHODS

For detailed descriptions see the corresponding paper.

PATIENT MATERIALS (Paper I-IV)

Bone metastases; castration-resistant, hormone-naïve (untreated) and short-term treated, were obtained from a series of fresh-frozen and formalin fixed paraffin embedded biopsies collected from patients with prostate cancer operated for metastatic spinal cord compression or pathologic fractures at Umeå University Hospital (2003-2011). From 16 patients we retrieved biopsies from the primary tumors and from two patients we had paired CT-guided vertebral needle biopsies taken immediately before and 3 days after surgical castration. In addition, we also used material from 13 patients who were treated with radical prostatectomy at Umeå University Hospital, between Feb 2005 and Sep 2006.

***IN VITRO* STUDIES**

Cell culture (Paper IV)

The 22Rv1 tumor cell line was used in functional experiments and chosen to represent CRPC with high AR activity and expression of AR-V7. Cells were cultured according to manufacturer´s recommendations (ATCC).

Yipf6 knock down, overexpression and functional evaluation (Paper IV)

Gene silencing was performed using the RNAi Human starter kit (Qiagen) according to manufacturer's instructions. siRNA specific for *YIPF6* was purchased as GeneSolution siRNA (Qiagen). YIPF6 was overexpressed in 22Rv1 cells using the TetOn 3G expression system (Clontech) as recommended. cDNA representing full length *YIPF6* and the shorter variant were amplified and cloned into the expression vector pTRE3G-ZsGreen 1 using In-Fusion PCR cloning technology (Clontech). *YIPF6* expression was induced with complete medium containing doxycycline at different concentrations (0, 10 and 100 ng/ml) for 48 to 72 hours. Proliferation of cells was analyzed using the Cell Proliferation Kit 1 (Roche). Migration and invasion abilities of the cells were studied using BD BioCoat control inserts and Growth Factor Reduced MATRIGEL Invasion chamber (BD Biosciences).

PROTEIN, RNA AND DNA PREPARATION (Paper II-IV)

Protein, RNA and DNA were extracted using the AllPrep DNA/RNA/Protein Mini kit or AllPrep RNA/Protein Mini kit according to the manufacturer´s instructions (Qiagen). RNA was in some cases extracted using the Trizol protocol (Invitrogen). Protein concentration was determined by the BCA Protein assay (Pierce Chemical Co), and DNA and RNA, by absorbance measurements using a spectrophotometer (ND-1000 spectrophotometer; NanoDrop Technologies). The RNA quality was analyzed with the 2100 Bioanalyzer (Agilent Technologies) and verified to have an RNA integrity number ≥ 6.

WESTERN BLOT (Paper II-III)

Samples were separated by 7.5 % SDS-PAGE under reducing conditions and subsequently transferred to PVDF membranes (Immobilon-P, Millipore). Membranes were blocked in 5 % milk followed by anti-AR antibody incubations; N-20 (diluted 1:500, Santa Cruz Biotechnology) or PG-21 (diluted 1:1000, Upstate) in order to detect the ARfl and AR-Vs, and C-19 (Santa Cruz) in order to detect ARfl but not AR-Vs lacking the LBD. Protein expression was visualized using secondary anti-rabbit IgG (Dako) antibody and an ECL Advanced detection kit (GE Healthcare) and quantified with a ChemiDoc scanner and the Quantity One 4 software (Bio-Rad laboratories).

MORPHOLOGICAL ANALYSIS

Immunohistochemistry (Paper I-IV)

Samples were frozen in liquid nitrogen or fixed in buffered formalin, decalcified in 20 % formic acid at 37°C for 1 to 3 days depending on the size of the specimen, and embedded in paraffin. Paraffin sections were stained with hematoxylin-eosin, and immunostained for AR (PG-21, Upstate), PSA (A0562, DAKO), activated caspase 3 (Cell Signaling), Ki67 (MIB1, DAKO), chromogranin A (5H7, Novacastra), AKR1C3 (Sigma) and YIPF6 (HPA003720, Atlas Antibodies). The cryostat sections were fixed in 4 % formaldehyde solution for 10 minutes and immunostained for AR (265M, Biogenex) following the protocol above except that antigen retrieval was not used.

The percentage of apoptotic (caspase 3 positive and cells showing the nuclear morphology of apoptosis in hematoxylin-eosin-stained sections) and proliferating (Ki67 positive) tumor epithelial cells were scored by evaluating 300-1000 cells per

patient. Nuclear AR and cytoplasmatic AKR1C3 and PSA staining in tumor epithelial cells were scored according to intensity (0 = no staining, 1 = weak, 2 = moderate, 3 = intense staining) and fraction of stained cells (1 = 1-25 %, 2 = 26-50 %, 3 = 51-75 %, 4 = 76-100 %). A total score (ranging from 0 - 12) was obtained by multiplying the staining intensity and fraction scores (distribution).

Immunofluorescence (Paper IV)

22Rv1 cells were seeded onto sterile ISO 8255 compliant cover glasses (Zeiss) and incubated for 3 days. The cells were formalin-fixed and stained with primary antibodies; anti-YIPF6 (HPA003720, Atlas antibodies) or anti-human Golgin-97 (A-21270, Molecular Probes) or isotype-matched control antibodies. Detection was made using fluorescent species-appropriate secondary antibodies (Alexa Fluor 568 monkey anti-rabbit IgG (H+L) or Alexa Fluor 647 anti-mouse IgG (H+L). The images were acquired on a Zeiss LSM 710 confocal microscope and analyzed with AxioVision (Carl Zeiss AB) or IMARIS (Bitplane AG, Zürich) software.

GENE EXPRESSION ANALYSIS

Real time RT-PCR (Paper II-IV)

cDNA was prepared by reverse transcription of total RNA with Superscript II or III reverse transcriptase (Invitrogen) according to protocol. Quantification of mRNA levels were performed using the Biorad iQ5 iCycler (Bio-Rad Laboratories) or the ABI PRISM 7900HT Instrument (Applied Biosystems) using the IQ SYBR Green Supermix (Bio-Rad laboratories) or the TaqMan gene expression mastermix (AppliedBiosystems, LifeTechnologies) according to manufacturers´ protocols. Each sample was adjusted for the corresponding *RPL13A* or *GAPDH* mRNA levels.

Gene expression array analysis (Paper II, IV)

Total RNA of each sample was used for cRNA production by the Illumina TotalPrep RNA amplification kit (Ambion) according to the provided protocol. The quality of cRNA was evaluated using the RNA 6000 pico kit (Agilent Technologies) and the Agilent 2100 Bioanalyzer (Agilent Technologies). Biotinylated cRNA was used for hybridization to a human HT12 Illumina Beadchip gene expression array (Illumina), including 48803 probes and 37846 annotated genes, according to the manufacturer's protocol. The arrays were scanned and fluorescence signals obtained using the Illumina Bead Array Reader (Illumina). Array data analysis was performed with GenomeStudio software

(Illumina). Samples were normalized by the cubic spline algorithm and differentially expressed genes were identified with the Mann-Whitney differential expression algorithm. Gene ontology analysis was done with the Metacore software (GeneGoInc) and IPA (Ingenuity ® Systems, www.ingenuity.com).

GENETIC ANALYSIS

Copy-number assay (Paper III-IV)

The copy number of the *AKR1C3, AR, YIPF6*, and *OPHN1* in PC bone metastases was examined using the TaqMan copy number assays (AppliedBiosystems); Hs03060693_cn (*AKR1C3*, exon 2) and the Hs02574521_cn (*AKR1C3*, exon 7), Hs00088448_cn (*AR*, exon 1), Hs04100122_cn (*AR*, intron 3), Hs00853200_cn (*YIPF6*), Hs00019056_cn (*OPHN1*). The number of X chromosomes was determined using the Hs02728251_cn assay targeting the X chromosome centromere region. The assays were run according to the manufacturer´s description, with RNaseP as reference gene, and analyzed using the Copy Caller software (AppliedBiosystems).

Fluorescence in situ hybridization (FISH) (Paper IV)

Sections of fresh frozen bone metastases were mounted on Super frost plus microscope slides (Thermo Scientific), fixed in absolute methanol:acetic acid (3:1) and in EtOH (70 %, 80 %, 95 %). *AR* signals were detected using an *AR* probe (SpectrumOrangeTM –labelled probe, locus Xq11-13, Vysis) and compared with signals for the X-chromosome detected with an alpha-satellite probe (SpectrumGreen-labelled CEP X, Vysis). A fluorescence microscope (ZEISS AXIO Imager Z1) was used to score signal copy numbers per nucleus for the *AR* and chromosome X. *AR* amplification was considered to be present when the average number of *AR* signals was more than five per cell, when the copy number of the *AR* was more than 2-fold higher than that of the CEPX, or when the amplification was easily recognizable such as in tight clusters with very high numbers of *AR* signals [44]. Gain of the X chromosome was defined as the presence of an equally elevated copy number for both *AR* and the CEPX reference probe and could thereby be clearly distinguished from gene amplification. At least 60 cells were scored per sample.

STATISTICS (Paper I-IV)

Correlations between variables were analyzed using Spearman rank test. Groups were compared using the Kruskal-Wallis followed by Mann-Whitney U test for continuous variables and the Chi-square test for categorical variables. Paired observations were compared using the Wilcoxon test. Kaplan-Meier survival analysis was performed with death of prostate cancer as event and follow-up time as time between metastasis surgery and the latest follow-up examination. Multiple Cox survival analysis was performed for evaluation of independent predictive variables. Statistical analyses were performed using the latest version of SPSS software. A P-value less or equal to 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

A summary of the results and discussion for each paper are listed below. More detailed data together with tables and figures can be found in the original articles of this thesis.

PAPER I

The mechanisms underlying castration resistance and growth of bone metastases in prostate cancer are still largely unexplained. Most of what we know is based on studies of primary tumors and soft tissue metastases and the reason to this is that clinical bone metastases are seldom resected. In paper I we analyzed bone metastasis tissue from previously untreated patients and from patients who had progressed after receiving ADT. By using immunohistochemistry we explored AR expression and possible down-stream effects of the AR such as cell proliferation, apoptosis and PSA expression in samples from altogether 60 patients with metastatic prostate cancer.

We found high nuclear AR immunostaining (a combined score of median or above; 8-12, referred to as "AR-high") in 28 (63 %) of the CRPC bone metastases. No difference in AR staining was observed between hormone-naïve and CRPC metastases, indicating reactivation of the AR in most cases of CRPC bone metastases. High AR immunostaining in CRPC metastases was found to be associated with a particularly poor prognosis after metastases surgery. This is similar to the association found between high expression levels of AR in tumor epithelial cells and early relapse after radical prostatectomy in primary prostate tumors [102, 103] and to the association between high AR levels in lymph node metastases and decreased cancer specific survival [104] and also to the fact that *AR* transcriptional activity is reactivated in most CRPC patients [35].

We then realized that the AR staining score in bone metastases was correlated to tumor cell proliferation, but not to tumor cell apoptosis or to PSA expression, indicating that nuclear AR staining does not necessarily mean that all possible down-stream targets are activated. In line with this, we observed divergences between nuclear AR immunostaining, PSA expression, and proliferation in specific tumor areas.

We also investigated the proportion of neuroendocrine (NE) cells in bone metastases since NE cells together with stem cells lack AR staining, and enrichment of those cell types could thus explain low AR levels in certain metastases. 16/44 CRPC bone metastases expressed chromogranin A (a marker for NE cells), but in most cases the fraction of NE tumor cells was very low. The metastases with a particularly high fraction of NE cells had a low AR staining score. However, tumor areas with low AR were also seen in metastasis areas lacking NE-cells. Low AR staining could probably not be explained only by the presence of NE-cells or stem cell-like tumor cells as such cell types in general are rare in bone metastases [32, 105], but could possibly be explained by the existence of adenocarcinoma cells by-passing the need for the AR.

In the clinic, changes in serum PSA levels are used to monitor effects of treatment in bone metastases. As suspected, high pre-operative serum PSA (above median; 190 ng/ml) at metastasis surgery was related to an unfavorable outcome in CRPC patients, probably by reflecting the total tumor burden. However, we found no correlation between serum PSA levels and the nuclear AR immunostaining which indicated that AR levels in the tumor cannot be predicted by measuring serum PSA. The lack of correlation can be due to the fact that prostate cancer metastases are heterogeneous both within and between different metastases, and the AR staining score in one tumor area might therefore not be the same as in another part of the tumor or in another metastasis.

Interestingly, a high PSA staining score (higher than median) in CRPC metastases was associated with a better outcome, in line with what previously has been observed both in metastases [32, 106, 107] and in primary tumors [11, 108], possibly by indicating some degree of tumor cell differentiation.

Although castration has been the standard treatment for prostate cancer bone metastases for the last 60 years, the short-term morphological effects of this treatment have not been examined in patients. In two cases we had access to paired biopsies taken before and 3 days after surgical castration. The castration induced decreased nuclear AR staining and an increase in tumor cell apoptosis, but only a moderate decrease in cell proliferation. This is different from previous studies of primary tumors were the cell proliferation were markedly reduced 3-4 days after treatment [11, 109, 110]. Different castration responses in primary and metastatic tumor

cells might be due to different therapy responses in the corresponding microenvironments. Castration in the prostate primarily works by affecting AR positive cells in the stroma and vasculature and only indirectly affects epithelial cells through reduction of stroma-secreted factors [109, 111], while the metastasis stroma is probably unaffected by castration therapy. Studies of the interactions between metastases tumor cells and the microenvironment and surrounding tissue are therefore important to be able to improve the treatment of patients with metastatic disease.

In conclusion, our study showed that the AR was reactivated in the majority of CRPC bone metastases examined and this seemed to be associated with a very poor clinical outcome. The mechanisms behind AR reactivation in CRPC metastases have not been thoroughly examined in clinical samples and further studies are therefore needed.

PAPER II

In paper I we found that intense nuclear AR immunostaining in CRPC bone metastases was related to a particularly short survival after metastasis surgery. This group of patients with suggested high AR activity could theoretically be successfully treated with drugs that block androgen synthesis or targets the AR. However, recent data published by others have indicated enrichment of constitutively active AR receptors during prostate cancer progression which might render the tumors resistant to androgen-deprivation therapy. Earlier reports have studied expression of AR-Vs in CRPC samples, but those samples were mainly obtained from primary tumors or soft tissue metastases and only a few bone metastases collected at autopsy have been examined. In this paper we examined the AR-Vs that so far had been reported as expressed in human tissue samples and therefore were thought to be the most clinically relevant forms. Therefore, the expression of the AR-Vs termed AR-V1, AR-V7 (AR3) and AR-V567es were studied in untreated, hormone-naïve (HN) and CRPC bone metastases in comparison to primary prostate cancer and non-malignant prostate tissue.

We detected the *AR-V1*, and *AR-V7* transcripts in most of the non-malignant, primary tumor, and metastases samples examined, while the *AR-V567es* transcript was detected in 7 (23 %) of CRPC bone metastases only. Clearly elevated *ARfl, AR-*

V1 and *AR-V7* transcript levels were seen in the CRPC bone metastases, which showed 8, 44, respectively 120 times higher median levels than the HN metastases. In contrast to Sun and co-workers [54], we found the *AR-V7* to be more commonly expressed than the *AR-V567es*. This disparity could possibly be technically explained by different sensitivities of the RT-PCR techniques used, but could possibly also reflect heterogeneities in AR splice variant expression between different tissue origins as we examined CRPC in bone while Sun *et al* [54] included CRPC from various sites.

High levels of constitutively active *AR-Vs* (*AR-V576es* and/or high levels of *AR-V7*, named AR-V high) in CRPC bone metastases were associated with high nuclear AR immunostaining score and furthermore related to short patients' survival. The poor prognosis of patients with CRPC metastases expressing high levels of *AR-Vs* was probably related to the postulated constitutive activity of these variants. Both AR-V7 and AR-V567es have been shown to translocate into the nucleus, bind to AR responsive elements, and activate/suppress gene transcription *in vitro* without the need for ligand binding [52-54, 58, 112].

We found higher levels of the AR splice variants in CRPC than in HN bone metastases, but we also detected the *AR-V1* and *AR-V7* transcripts in a substantial part of the non-malignant and malignant radical prostatectomy specimens, although at lower levels than in the bone metastases. This raises the question whether AR-Vs are increased as a result of castration treatment, and thereby contribute to relapse from therapies aiming to reduce steroid levels or ligand binding to the normal AR, or could be part of the normal prostate physiology and that a selection of AR-Vs occurs during prostate cancer progression. In a xenograft model for prostate cancer, *AR-V7* and *AR–V1* mRNA levels were increased after castration, as well as a decreased after androgen supplementation, indicating that some AR-Vs may be directly regulated by androgens and thus probably induced in patients shortly after castration therapy [58]. Moreover, others have shown that increased expression of AR-V7 in primary prostate tumors was associated with shorter time to disease relapse after radical prostatectomy [52, 53].

To examine what characterized metastases with high AR-Vs at the molecular level, we used a whole-genome expression array and compared the expression profile in the AR-V high bone metastases to the profile in the other CRPC bone metastases.

We found that metastases with high *AR-V* expression seemed to be associated with differentiating gene products which were directly interacting via *AR, C-MYC,* and *CDK1* and thus indicated a disturbed cell cycle control. However, we could not see any significant difference in Ki67 immunostaining between the two groups of metastases. Based on our data, we were not able to discriminate if the disturbed cell cycle control seen in the AR-V high bone metastases was truly related to the expression of the AR-Vs, the ARfl, or just associated with the particularly advanced disease in those CRPC cases. However, a study published after this paper where LNCaP cells were transfected with AR-V7 and AR-V567es showed that AR-Vs had a unique role in the activation of cell cycle genes that did not require the presence of full-length AR, and cell cycle genes such as *ZWINT, UBE2C* and *CCNA2* have been associated with AR-V expression also in other studies [113, 114]. But further studies are needed to fully establish if AR-Vs and AR-fl regulate an overlapping yet distinctive set of target genes, and if so, are there any suitable ways to target signaling down-stream of AR-Vs?

We further studied the protein levels of the AR-Vs in 13 CRPC bone metastases samples by using western blot. Two antibodies were used that targeted the N-terminal domain and the C-terminal LBD domain of the AR, respectively. The highest of an 80 kDa LBD-truncated protein levels were detected in samples with high *AR-V7* mRNA levels (levels in the upper quartile, Q4). The CRPC bone metastases with lower *AR-V7* mRNA levels showed low to undetectable protein levels regardless of corresponding *AR-V567es* mRNA levels. Most importantly, we noted that there was a significant discrepancy in the relative levels of AR-Vs measured at the RNA level compared to those observed at the protein level. Whereas mRNA levels of the variants showed median levels ranging between 0.4 %- 1 % relative to levels of the full length AR, the relative median expression at the protein level, derived from western blot analysis, was 32 %. Those results might indicate that AR-Vs lacking the LBD domain are post-transcriptionally stabilized in selected CRPC bone metastases, and furthermore that the AR-Vs are expressed at levels similar to the full-length AR in some CRPC patients and thus probably are of high clinical relevance in those individuals. Reasons for this suggested AR-V stabilization at the protein levels are unknown, and need to be further studied.

PAPER III

Patients expressing high levels of AR-Vs (paper II) probably show poor response to androgen deprivation therapy [115] and anti-AR drugs targeting the LBD. However, some CRPC patients respond to 2nd line ADT and this might be due to intra-tumoral steroidogenesis and production of testosterone and DHT which is another suggested mechanism behind CRPC. Several studies have reported increased levels of steroidogenic enzymes in CRPC tumors [65, 69, 70]. However, it is not known if these enzymes are upregulated already in previously untreated HN metastases or as a result of castration treatment like the AR-Vs, studied in paper II.

Tissue androgens might be derived by *de novo* synthesis from cholesterol [67, 68] or by conversion of adrenal precursors into more potent steroids [66]. We used RT-PCR to study the expression levels of key enzymes involved in the formation of testosterone and DHT from cholesterol (Figure 3). Surprisingly, none of the analyzed enzymes in the steroidogenesis pathway showed significantly different mRNA expression levels between untreated HN (n = 9) and CRPC bone metastases (n = 45).

In line with a previous report [70], but in contrast to others [65, 69], we found that transcript levels of enzymes in the early steps of steroidogenesis; *CYP11A1, CYP17A1* and *HSD3B2,* showed low expression levels in both untreated and CRPC metastases. High mRNA levels of these enzymes were instead found in non-malignant prostate tissue, probably due to high synthesis of those enzymes in normal prostate stromal cells, and the same was true for *SRD5A2* and *HSD17B6* [116-119]. However, the *CYP11A1, CYP17A1* and *HSD3B2* mRNA levels were found to be highly correlated in CRPC bone metastases.

In contrast to the generally low levels of enzymes corresponding to the early steps of steroidogenesis, we found high transcript levels of enzymes involved in the later steps of steroid synthesis; *SRD5A1, AKR1C2, AKR1C3*, and *HSD17B10* in bone metastases when compared to non-malignant and malignant prostate tissue. The high mRNA levels of *AKR1C3* and *SRD5A1* in metastases were in line with previous reports [65, 69, 70] and may indicate synthesis of testosterone and DHT from adrenal-derived androstenedione. By the activity of SRD5A1, DHT could also be synthesized via 5α-reduction of androstenedione to androstanedione and then by further reduction of androstanedione to DHT by AKR1C3. This route for DHT

synthesis, which bypasses testosterone, has been shown to dominate in CRPC cell lines as well as in patient metastases specimens when stimulated with androstenedione [120].

Taken together we think that these results indicate that CRPC bone metastases may have an induced capacity of converting adrenal-derived androgens into more potent ones, while *de novo* synthesis of androgens from cholesterol is less likely, except maybe in individual cases with correlated expression of CYP11A1, CYP17A1, and HSD3B2.

Although none of the examined steroidogenic enzymes showed significantly increased expression levels in CRPC compared to HN bone metastases, individual CRPC metastases expressed very high transcript levels. A subgroup of CRPC metastases showed high levels of *AKR1C3* mRNA as well as correlated protein levels according to IHC analysis. The high AKR1C3 protein levels in a subgroup of prostate cancer bone metastases confirmed results previously reported for CRPC tissue of different metastatic origin [65, 69, 121]. A rise in AKR1C3 activity could contribute to the conversion of adrenal-derived steroids into more potent AR ligands, as discussed above, and to AR activation in CRPC. However, we could not see that high AKR1C3 protein expression was significantly linked to AR activity in CRPC bone metastases (AR immunostaining score, paper I). These results suggest that AKR1C3 probably has additional roles in metastases apart from enhancing steroid conversion.

Then we analyzed the AKR1C3 protein levels in relation to expression of AR-Vs, in order to identify potential different mechanisms behind CRPC in individual bone metastases. The ARfl and AR-V protein levels were studied by western blot in 29 of the CRPC bone metastases, and it was observed that high protein levels of AR-Vs were found in bone metastases with low AKR1C3 levels, while metastases with high AKR1C3 levels primarily contained low AR-V levels. The possible clinical relevance of this is that patients with high AKR1C3 expression might theoretically be patients showing good response to treatment with abiraterone acetate (CYP17 inhibition [122]) and/or would benefit from drugs targeting AKR1C3 [123], while patients with high expression of constitutively active AR-Vs probably will not respond to abiraterone acetate or to any therapy targeting androgen synthesis or the LBD of the AR.

In conclusion, we think that increased tumor expression of steroidogenic enzymes in individual patients is not clearly associated with CRPC but merely associated with advanced tumor stage, as it can be seen in both CRPC samples and in previously untreated, hormone-naïve, bone metastases. A subgroup of metastases expressed very high levels of AKR1C3, indicating that this group may have an induced capacity of converting adrenal-gland derived steroids into more potent androgens.

PAPER IV

In paper II and III we have studied two of the possible mechanisms contributing to castration-resistance in bone metastases; intratumoral steroidogenesis and expression of AR splice variants. Another mechanism behind CRPC and reactivation of AR is *AR* amplification. The relation between *AR* amplification, expression of AR splice variants and steroid-converting enzymes in CRPC has however been poorly examined. In this paper we examined *AR* amplification in hormone-naïve and CRPC bone metastases and explored molecular and functional consequences of *AR* amplification.

By using FISH and copy number analysis we found *AR* amplification in none of the HN, untreated bone metastases (n = 10) but in 16/30 (53 %) of the CRPC metastases, which was more frequent than previously reported for bone metastases [124]. Furthermore, we saw that bone metastases with *AR* amplification had increased *AR* mRNA levels, but was also associated with high levels of the constitutively active *AR-V7* splice variant. However, high *AR-V7* levels were not exclusively found in metastases with *AR* amplification, but also in a few cases with no detectable *AR* amplification.

To examine possible molecular characteristics in the bone metastases with *AR* amplification, we used data from a whole-genome cDNA array analysis (paper II) and compared the expression profile between CRPC metastases with and without *AR* amplification. We found that *YIPF6* which is located about 1 MB from the *AR* gene on the X chromosome was highly expressed in the metastases with *AR* amplification. The *YIPF6* expression levels correlated to copy numbers of the gene and *YIPF6* was thus found to be coamplified with the *AR*. By using confocal microscopy we could see that YIPF6 was localized to the Golgi apparatus, which is the same to what has been reported for other YIP family members [125]. Further

studies of YIPF6 function in prostate cancer are needed to find out if YIPF6 has an important role in bone metastases with *AR* amplification.

Ontology gene analysis indicated that many of the genes differentially expressed between bone metastases with and without *AR* amplification seemed to be involved in processes of bone growth and remodeling. Interestingly, several genes that are known to directly or indirectly stimulate osteoclast differentiation and activity [126] were down-regulated in the group of metastases with *AR* amplification, indicating impaired osteoclast activity in those cases. It is known that prostate cancer generally form osteoblastic/sclerotic bone metastases but the pathophysiology and development of these metastases is not fully understood. Our results indicating lower osteoclast activity with increasing AR activity is thus of high biological interest, and further strengthened by a recent study from our group where AR positive LNCaP cells were shown to stimulate a sclerotic response when co-cultured with calvarian bone, while AR negative PC-3 cells stimulated osteoclast activity and bone resorption [127].

Taken together our data indicate that bone metastases harboring *AR* amplification have a more sclerotic phenotype than other metastases, that could be caused by reduced osteoclast activity and reduced bone resorption and that might be associated with high AR activity. However, the present findings are limited to data at the RNA level, and results and conclusions need to be verified both at protein and functional levels.

CONCLUSIONS

- High nuclear AR expression is found in the majority of bone metastases in CRPC patients, and this is associated with poor prognosis

- Expression of AR splice variants are increased in CRPC compared to hormone-naïve bone metastases and is related to short patients' survival

- Protein expression of AR splice variants can be found at levels comparable to levels of the full length AR in CRPC

- A subgroup of prostate cancer bone metastases might have an induced capacity of converting adrenal-gland derived steroids into more potent androgens

- Subgroups of bone metastases could be identified according to their expression levels of AKR1C3, AR-Vs and *AR* amplification, which might be of relevance for patient response to 2nd line androgen-deprivation therapy

- *AR* amplification in CRPC bone metastases seems to be associated with decreased osteoclast activity, and consequently with decreased bone resorption and increased bone mineral density

GENERAL DISCUSSION

The majority of prostate cancer patients progress after ADT and develop CRPC predominantly as bone metastases. Most of our current knowledge about prostate cancer and mechanisms behind treatment failure is unfortunately based on studies of primary tumors or soft tissue metastases, and not on studies of metastases in the clinically most relevant site, the bone. When mechanisms behind metastasis growth have been the subject for research, studies have mainly been done in experimental models and not in patient materials.

Novel findings presented in this thesis

The probably most important finding in this thesis is that we, for the first time, show that constitutively active AR splice variants are frequently expressed in CRPC bone metastases, enriched at the protein level and therefore probably of high clinical relevance. We also highlight the possible importance of AKR1C3 (which earlier has been discussed as a potential target in CRPC) in a subgroup of bone metastases, and the possibility of using this enzyme as a therapeutic target. Moreover, we would like to emphasis that CRPC bone metastases with *AR* gene amplification seem to have decreased osteoclast activity, and consequently decreased bone resorption.

Biological interpretation

Like us, several groups have reported that CRPC tumors, compared with primary prostate cancer or normal prostate tissue, exhibit increased expression of enzymes involved in androgen synthesis. High AKR1C3 protein levels not only in CRPC metastases with high AR levels but also in hormone-naïve metastases and in metastases with low nuclear AR immunostaining, as demonstrated here, also suggests that AKR1C3 has additional roles apart from steroid conversion in prostate cancer bone metastases. AKR1C3 possesses prostaglandin F synthase activity which could enhance proliferation in prostate cancer cells independent of androgen and AR status [128], *i.e.* in hormone-naïve as well as CRPC metastases.

We noted that focal areas in primary prostate tumors could have as high expression of the AKR1C3 protein as was seen in certain bone metastases. One could speculate that prostate cancer cells with high AKR1C3 levels have a growth

advantage compared to cells with low enzyme levels and therefore are able to metastasize and grow in the bone. Otherwise, expression of steroidogenic enzymes in previously untreated bone metastases could possibly be promoted by factors in the bone microenvironment. Furthermore, steroidogenic enzymes are known to be expressed in tumor epithelial cells but also been reported to be expressed in the stroma and vasculature and we found AKR1C3 to be highly expressed in endothelial cells. Therefore, it is possible that steroid synthesis occurs both within the prostate cancer cell and outside the cell in the (bone) stromal compartment.

The mechanism behind the enrichment of AR-Vs in CRPC is not completely understood but one could speculate that *AR* amplification and the subsequent increase of *AR* transcripts might rise the probability for alternative *AR* splicing to occur. However, high *AR-V7* levels were not exclusively seen in metastases with *AR* amplification, but also in a few cases with no detectable *AR* amplification. In a xenograft model for prostate cancer, *AR-V7* mRNA levels were increased after castration, as well as decreased after androgen supplementation, indicating that AR-Vs could be directly regulated by androgens. Furthermore, intragenic *AR* deletions have been suggested to enhance AR-V7 and AR-V567es expression in CRPC [129-131]. If *AR* amplification or other structural changes in the *AR* gene are required for enrichment of AR-Vs during CRPC are today not fully understood. Also in CRPC, transcript levels of *AR-Vs* are quite low compared to full length *AR* and therefore, their functional significance have been questioned. However, several of the CRPC bone metastases examined in this thesis expressed LBD-truncated AR proteins at levels comparable to the ARfl protein levels, even though the corresponding *AR* variant transcripts were found at relatively much lower levels than the *ARfl* mRNA. This might indicate that AR-Vs lacking the LBD domain are post-transcriptionally stabilized in selected CRPC bone metastases in relation to the ARfl, and furthermore, that the AR-Vs in some CRPC patients are expressed at levels similar to the full-length AR and thus probably are of clinical relevance. Reasons for this suggested AR-V stabilization at the protein level as well as the mechanisms behind enrichment of *AR-V* transcripts are unknown, and need to be further studied.

The pathophysiology behind the sclerotic phenotype of prostate cancer bone metastases is not completely understood. Our findings suggesting low osteoclast

activity in *AR* amplified bone metastases may be of high biological interest, but requires further examination.

Potential clinical importance

We have identified subgroups of bone metastases according to their expression levels of AKR1C3 and AR-Vs, and *AR* amplification status, which we theoretically think will show diverse responses to different modes of therapies for CRPC. This hypothesis need to be further studied in the clinic, by evaluating patients' response to therapies for CRPC in relation to expression of AKR1C3 and AR-Vs.

One could speculate that patients with high AKR1C3 and AR metastases levels are dependent on steroidogenesis and therefore would show good response to drugs targeting the androgen synthesis, like abiraterone. However, recent studies in prostate cancer xenografts have shown up-regulation of AKR1C3 after treatment with abiraterone [132, 133] and those patients may benefit from further treatment with AKR1C3 inhibitors [123]. Compared to abiraterone, AKR1C3 inhibitors act downstream in the steroid synthesis and will not, like abiraterone, have side effects in form of accumulation of mineralocorticoids [134]. As indicated above, AKR1C3 inhibitors may also have growth inhibitory effects in tumors independent of AR-status, via its ability to inhibit prostaglandin F synthase activity.

AR-Vs have recently been shown to drive resistance to both enzalutamide (MDV3100) [113, 135] as well as to abiraterone acetate [113, 132] in prostate cancer cell lines and CRPC xenografts and it is therefore likely that the AR-Vs will not respond to 2nd line ADT targeting the androgen synthesis or the LBD of the AR. The constitutively active AR-Vs could possible instead be treated with drugs targeting the AR NTD or their down-stream targets [136, 137]. They may also be good targets for taxanes due to inhibition of both mitosis and AR translocation [42].

The AR-V567es variant has been shown to induce androgen-independent invasive adenocarcinoma in an AR-V567es transgenic mouse which indicates its potency in tumorigenesis, but its relevance in patients is still uncertain. In contrast to high levels of *AR-V7*, high *AR-V567es* mRNA levels did not correspond to a strong 80 kDa band (representing the AR-Vs) in western blot analysis of patient samples, and we therefore think that the AR-V7 is clinically more important.

To be able to monitor possible biomarkers, such as AKR1C3 and AR-V7, in patients we need to find ways that could reflect the whole tumor burden in prostate cancer patients. As it is difficult to receive tissue biopsies from multiple metastatic sites from each patient, presence of AR splice variants and AKR1C3/steroidogenic enzymes could possibly be measured in circulating tumor cells [121] or maybe also in exosomes [138, 139] and blood platelets/thrombocytes [140] from prostate cancer patients.

Weaknesses

The work in this thesis is based on a limited number of bone metastases, and a single metastatic site for each patient which make conclusion regarding this heterogeneous disease and over all patient status uncertain. High nuclear AR immunostaining was in this thesis presumed to reflect high AR activity in the bone metastases, but localization of AR in the nucleus does not necessarily mean that AR signaling is active. AR regulated gene transcription is affected not only by AR translocation into the nucleus and binding to DNA, but also by the recruitment of specific coregulators. Changes in the expression of different coregulators could possibly explain the observed divergences between AR and PSA expression, and proliferation in specific tumor areas [93, 141], and further studies are needed to evaluate this. We have studied three of the most abundant AR-Vs found in clinical samples so far, but it is of course possible that additional AR variants exists that contribute to drug resistance in CRPC. Furthermore, mRNA expression and protein levels of steroidogenic enzymes in bone metastases may not necessarily reflect biological activities.

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