Molecular Heterogeneity of Prostate Cancer Bone Metastasis

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“One of the painful things about our time is that those who feel certainty are stupid, and those with any imagination and understanding are filled with doubt and indecision.”

– Bertrand Russell
# Table of Contents

Abstract .......................................................................................................................... iii  
Populärvetenskaplig sammanfattning ........................................................................... v  
Abbreviations ................................................................................................................ vii  
Original papers ............................................................................................................. ix  

## Introduction  .................................................................................................................. 1

- Prostate Cancer ......................................................................................................... 1
  - Diagnosis ................................................................................................................ 2  
  - Bone metastasis .................................................................................................... 2  
  - Treatment and prognosis ...................................................................................... 4  
  - Androgen deprivation therapy ............................................................................. 5  
- Androgen receptor signaling ...................................................................................... 6  
- Castration-resistant prostate cancer ......................................................................... 9  
  - Treatment of castration-resistant prostate cancer .............................................. 9  
  - Mechanisms behind castration-resistant prostate cancer ................................. 11

## Aims ............................................................................................................................. 15

- Overall aim ............................................................................................................... 15  
- Specific aims ............................................................................................................. 15
  - Paper I .................................................................................................................. 15  
  - Paper II ................................................................................................................ 15  
  - Paper III .............................................................................................................. 15  
  - Paper IV .............................................................................................................. 15

## Materials and methods ............................................................................................ 16

- Patient materials (paper I-III) .................................................................................. 16  
- Tissue preparation (paper I-III) .............................................................................. 16  
- Extraction of RNA, DNA and Protein (paper I-IV) ................................................ 17  
- RNA analysis ........................................................................................................... 17
  - Quantitative real-time RT-PCR (paper I) ............................................................ 17  
  - Whole-genome expression arrays (paper I-IV) .................................................. 17
Abstract

Castration-resistant prostate cancer (CRPC) develops after androgen deprivation therapy of advanced prostate cancer, often with metastatic growth in bone. Patients with metastatic CRPC have very poor prognosis. Growth of CRPC, in most but not all patients, seems to involve androgen receptor (AR) activity, despite castrate levels of serum testosterone. Multiple mechanisms behind AR activation in castrated patients have been described, such as AR amplification, AR mutations, expression of constitutively active AR variants, and intra-tumoral steroid synthesis. However, other mechanisms beside AR activation are also involved and CRPC patients with tumors circumventing the need for AR stimulation will probably not benefit from AR targeting therapies but will need alternative treatments.

Available treatments for CRPC are chemotherapy, AR antagonists or inhibition of androgen-synthesis. Novel drugs are constantly under development and several new therapies has recently been approved for clinical use. These include, in addition to new AR targeting therapies also immunotherapy, osteoclast inhibitors and bone-targeting radiopharmaceuticals. Due to heterogeneous mechanisms behind CRPC and that newly developed therapies are based on different mechanisms of action, there are reasons to believe that CRPC patients show different therapy responses due to diverse molecular properties of individual tumors. Although there are promising prospects, no biomarkers are used today for patient stratification into different treatments. Another important aspect is that, when effective, any therapy will probably induce tumor responses that subsequently cause further molecular diversities and alternative paths for development of tumor relapse and castration-resistance. Such mechanisms are important to understand in order to develop new treatment strategies.

In this thesis, global gene expression and methylation patterns were studied in bone metastases obtained from prostate cancer patients going through metastasis surgery for spinal cord compression. Gene expression patterns were analyzed by multivariate statistics and ontology analysis with the aim to identify subgroups of biological/pathological relevance. Interesting findings from array analysis were verified using qRT-PCR and immunohistochemical analysis. In addition, a xenograft mouse model was used to study the effects of abiraterone (steroidogenesis inhibitor) and cabazitaxel (taxane), and subsequently developed resistance mechanisms in the 22Rv1 prostate cancer cell line expressing high levels of AR-V7; a constitutively active AR splice variant associated with a poor prognosis and resistance to AR targeting therapies.
In summary, results showed that the majority of CRPC bone metastases were AR-driven, defined from high levels of AR-regulated gene transcripts, while a smaller sub-group was non-AR-driven (paper I). AR-driven bone metastases had high metabolic activity in combination with downregulated immune responses while non-AR-driven cases had a more pronounced immune response (paper I) and higher bone cell activity (paper II). Paper III identified pronounced hypermethylation in primary prostate tumors probably causing a suppressed anti-tumor immune-response whereas metastases showed a different methylation pattern related to increased AR activity and patient outcome. In paper IV, 22Rv1 xenografts showed poor response to abiraterone and initially excellent response to cabazitaxel, but eventually resistance occurred probably due to an upregulation of the ABCB1 transporter protein. Anti-androgens partly reversed the resistance.

In conclusion, we have identified molecular heterogeneities in clinical bone metastases associated with biological characteristics, which could perhaps be used both for stratifying patients into treatment modalities, and to aid in further development of effective therapies for CRPC.
Prostatacancer är den vanligaste förekommande cancerformen hos män i Sverige och övriga västvärlden. Varje år diagnosticas ca 10 000 svenska män med prostatacancer varav en knapp fjärdedel kommer att avlida till följd av sjukdomen. Avancerad prostatacancer är när cancern har spritt sig utanför prostatan och bildat så kallade metastaser, vilket främst sker i skelettet. Standardbehandling vid avancerad prostatacancer är olika typer av kastrationsbehandling, även kallat för hormonell behandling, vilken syftar till att strypa tillförseln av androgener, manliga könshormoner, som binder till och aktiverar androgenreceptorer inuti cancercellerna.

Initialt är kastrationsbehandlingen en mycket effektiv behandling som bromsar upp tumörtillväxten och lindrar sjukdomen. Olyckligtvis så är effekten bara temporär och håller maximalt under några år innan cancern på något sätt lyckas kringgå kastrationsbehandlingen för att utvecklas vidare till ett obotligt stadium med mycket dålig prognos som kallas för kastrationsresistent prostatacancer. Utvecklingen av kastrationsresistent prostatacancer är ett mycket intensivt forskningsområde och även om de bakomliggande orsakerna ännu inte är helt klarlagda så har man sett att återaktivering av androgenreceptorn är involverad i de flesta fall, dock inte alla. Detta trots att nivåerna av androgener i blodet fortfarande är mycket låga till följd av kastrationsbehandlingen. Det finns flera olika föreslagna mekanismer som t.ex. amplifiering av androgenreceptorn, muterade androgenreceptorer, ligandoberoende varianter av androgenreceptorn och intratumoral steroidsyntes.

Tillgängliga behandlingar mot kastrationsresistent prostatacancer innefattar bland annat cytostatika, androgenreceptor-antagonister och inhibering av steroidsyntesen. Det sker en ständig utveckling av nya behandlingsalternativ och flera nya läkemedel har på senare tid blivit godkända för kliniskt bruk. Utöver nya behandlingar riktar mot androgenreceptorn och androgensignalering innefattar det även immunterapi och behandling med radioaktiva isotoper som söker sig till bennmetastaser för att där ge en lokal strålningseffekt. Då flera heterogena mekanismer tros ligga bakom utvecklingen av kastrationsresistent prostatacancer och det faktum att de nyutvecklade behandlingarna baserar sig på olika verkningsmekanismer kan man tänka sig att patienterna kommer uppvisa olika behandlingssvar på grund av olika molekylära egenskaper hos enskilda tumörer. Idag finns det inga behandlingsprediktiva markörer som används för att välja vilka typ av behandling man ska ge den enskilda patienten. En annan viktig aspekt är att även behandlingar som initialt är effektiva kommer så småningom sannolikt att leda till att tumörcellerna utvecklar nya resistensmekanismer vilka är viktiga att förstå för utveckling av nya behandlingsstrategier.
I den här avhandlingen har vi med hjälp av flertalet molekylärbio logiska metoder och multivariata dataanalyser studerat genexpressionsmönster och epigenetiska förändringar (DNA-metylering) i vävnadsprover från patienter med benmetastaserad prostatacancer som genomgått kirurgisk behandling av metastatisk ryggmärgskompression. Genexpressionsmönster analyserades med målet att identifiera biologisk och/eller patologiskt relevanta subgrupper av benmetastaser. Vi har även använt oss av en xenografit musmodell för att studera effekten av abiraterone (steroidsyntes-hämmare) och cabazitaxel (cytostatika) i prostatacancer celllinjer som uttrycker AR-V7, en ligandoberoende variant av androgenreceptorn som associeras med en mycket dålig prognos och en sannolik resistens mot terapier som riktar sig mot androgenreceptorn.

Resultaten visar att en majoritet av benmetastaser från patienter med kastrationsresistent prostatacancer drivs av androgenreceptor-aktivitet, baserat på ett högt genuttryck av gener som regleras av androgenreceptorn, medan en mindre subgrupp inte verkar vara drivna av androgenreceptorn (arbete I). Benmetastaserna som drivs av androgenreceptorn visade sig även ha hög metabol aktivitet och en nedreglerad cellulär immunrespons (arbete I) medan de benmetastaser som inte drivs av androgenreceptorn uppvisade ett mer uttalat cellulärt immunvärk och högre aktivitet av benbildande celler (arbete II). Arbete III visar en tydligt ökad metyleringsgrad under progressionen av prostatacancer, möjligtvis relaterat till en undertryckt immunfenotyyp, och metastaserna visade olika metyleringsmönster relaterade till androgenreceptor-aktivitet och prognos. I arbete IV så svarade xenografitmodellerna dåligt på behandling med abiraterone medan behandling med cabazitaxel visade sig initialt vara mycket effektiv. Så småningom uppstod dock resistens även mot cabazitaxel genom en uppreglering av transportproteinet ABCB1 och behandling med anti-androgen visade sig kunna partiellt motverka denna resistensmekanism.

Sammanfattningsvis så har vi i denna avhandling identifierat molekylära heterogeniteter i benmetastaser från patienter med prostatacancer som är associerade med biologiska egenskaper som eventuellt skulle kunna användas till behandlingsstratifiering när patienter ska väljas ut till olika typer av behandlingar och även potentiellt bidra till utvecklingen av nya behandlingar mot kastrationsresistent prostatacancer.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>4-dione</td>
<td>Androstenedione</td>
</tr>
<tr>
<td>ABCB1</td>
<td>ATP-binding cassette sub-family B member 1</td>
</tr>
<tr>
<td>ADT</td>
<td>Androgen deprivation therapy</td>
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<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>AREs</td>
<td>Androgen response elements</td>
</tr>
<tr>
<td>AR-V567es</td>
<td>Androgen receptor variant 567es</td>
</tr>
<tr>
<td>AR-V7</td>
<td>Androgen receptor variant 7</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic proteins</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostate hyperplasia</td>
</tr>
<tr>
<td>Ca2+</td>
<td>Calcium</td>
</tr>
<tr>
<td>CAB</td>
<td>Combined androgen blockade</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CE3</td>
<td>Cryptic exon 3</td>
</tr>
<tr>
<td>CRPC</td>
<td>Castration-resistant prostate cancer</td>
</tr>
<tr>
<td>CTCs</td>
<td>Circulating tumor cells</td>
</tr>
<tr>
<td>CTD</td>
<td>COOH terminal domain</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte–associated antigen 4</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DM-CpGs</td>
<td>Differentially methylated CpG sites</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRE</td>
<td>Digital rectal exam</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>ERα</td>
<td>Estrogen receptor α</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin-embedded</td>
</tr>
<tr>
<td>FGFs</td>
<td>Fibroblast growth factors</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GSEA</td>
<td>Gene set enrichment analysis</td>
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<tr>
<td>HSP</td>
<td>Heat shock protein</td>
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Original papers

This thesis is based on the following papers, referred to by their roman numerals:


†To be regarded as joint first authors
Other papers I have participated in during my doctoral education (not included in thesis)


Introduction

Prostate Cancer

Prostate cancer is the most common cancer type and a leading cause of cancer mortality amongst men in Sweden and many other developed countries [1]. The incidence has steadily increased for many years and approximately a third of all cancer in Swedish men is prostate cancer. In 2016, 10,473 Swedish men were diagnosed with prostate cancer and 2,347 died because of the disease. Prostate cancer is a disease that occurs mainly in older men, the median patient is 70 years old at diagnosis, but in some cases the disease is diagnosed in men less than 50 years old (The National Board of Health and Welfare, Sweden). Prostate cancer usually does not give rise to any noticeable symptoms until the disease has entered a more advanced stage. One factor contributing to the increased incidence is the development of the serum prostate specific antigen (PSA) test introduced during the late 1980’s which proved better at early detection than digital rectal exam (DRE) [2-5].

The prostate is a gland that surrounds the upper part of the urethra and is located just below the urinary bladder in front of the rectum. Its main function is to secrete prostate fluid, which is included in the ejaculate. The major protein within the prostate fluid is PSA, a protease that helps the semen to be liquefied. In healthy men, PSA is secreted into the prostate lumen by prostate epithelium, transported to the urethra and discharged during ejaculation [6]. The elevated levels of PSA in serum blood is caused by the tumor disturbing in the normal prostate architecture leading to PSA leaking out of the prostate epithelium. However, raised PSA can also be caused by non-malignant conditions such as prostatitis and benign prostate hyperplasia (BPH) which means that elevated serum-PSA is not equal to prostate cancer [7, 8]. The PSA test has also received a lot of criticism because it is considered to lead to overdiagnosis and overtreatment of patients who because of small, slow-growing tumors are unlikely to experience clinical symptoms within their lifetime. [9].

Despite the risk of overdiagnosis and overtreatment, many patients with prostate cancer are diagnosed and treated to late allowing the disease to progress into an incurable stage where the cancer have spread outside the prostate, often in the form of bone metastases [10]
**Diagnosis**

Despite the ongoing debate, serum PSA is still an important tool for prostate cancer risk assessment and, together with a physical examination in the form of DRE, it is used when prostate cancer is suspected. The so called normal PSA value varies between individuals and tend to increase with age. In Sweden the limit is <3ng/µL for men up to 70 years, <5ng/µL for 70-80 years and <7ng/µL for >80 years, a value exceeding this is considered to give reasons for further investigation (National Prostate Cancer Care Program, Sweden). After further clinical evaluation patients might be subjected to ultrasound or MR guided needle biopsies of the prostate. These biopsies are used for histological examination where eventual tumors are graded according to the Gleason system [11]. It is the standard system used to predict prostate cancer prognosis, although it has been modified and complemented since first constructed in the mid-1960’s [12]. If prostate cancer is detected, an investigation, using imaging methods and bone scintigraphy, is made to determine the spread of the cancer. Localized prostate cancer is confined within the fibrous capsule largely covering the prostate gland and is stratified into different risk groups (very low, low, intermediate and high risk) based on tumor size/extent, Gleason score and serum PSA. A cancer that is spread outside the prostate, but with no signs of distant metastases, is considered as a locally advanced prostate cancer. If metastases is found in distant organs it is considered as advanced prostate cancer (National Prostate Cancer Care Program, Sweden).

**Bone metastasis**

In most men with advanced prostate cancer the disease have metastasized to bone and bone metastases are found in the majority of men dying due to prostate cancer. Metastases can also be found at other sites such as lymph nodes, lungs and liver [13, 14]. Bone metastases are most commonly found in sites with hematopoietic (red) bone marrow such as the vertebral column, pelvis, ribs, femurs and skull. Common complications are bone pain, spinal cord compression and pathological fractures [15].

There are two major theories explaining why cancers tend to favor certain sites when they metastasize [16]. The first was proposed by the English surgeon Stephen Paget in 1889 and came to be known as the “seed-and-soil hypothesis”. He symbolized the metastatic cancer as a plant that sows its seeds and that even though the seeds (tumor cells) are carried in all directions they can only grow if they fall on congenial soil (a favorable microenvironment). In order for metastases to form, the tumor cells must be compatible with the microenvironment [17]. In 1928 came a second theory by James Ewing, the “hemodynamic hypothesis”. It challenged Paget’s theory by stating that distribution of metastases will be determined by the anatomical structure of the
vascular and lymphatic drainage. According to Ewing’s hypothesis metastases are formed when tumor cells arrest nonspecifically, the first organ encountered will be the primary site of tumor arrest and will therefore have the highest number of metastases [18]. Based on this theory, the fact that blood from the prostate is drained into intraspinal veins, via a venous structure called Batsons venous plexus, would explain why prostate cancer metastases often arise in the spine [19]. Even though Ewing’s hypothesis prevailed for several decades, today the consensus is that those hypotheses are not mutually exclusive instead both might be true to some extent. Regional metastases could be determined by anatomical or mechanical factors while distant metastases are more site specific [20].

Why prostate cancer metastasize to bone might be explained by several factors; blood flow is high in hematopoietic bone marrow and tumor cells express adhesion molecules binding them to bone matrix and stromal cells within the bone marrow, a rich environment where the tumor cells can get access to various growth factors such as transforming growth factor betas (TGF-βs), insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), bone morphogenetic proteins (BMPs), endothelin-1 (ET-1) and calcium (Ca²⁺) and others involved in supporting hematopoiesis. Bone is a dynamic tissue that maintains its structural integrity through a constant state of remodeling. In normal bone remodeling, there is a balance between resorption of old bone, by osteoclasts, and formation of new bone, by osteoblasts. When tumor cells colonize the bone this balance is altered [21]. Prostate cancer often form bone metastases which are generally classified as osteoblastic (also called sclerotic), with increased bone formation, in contrast to many other osteotropic cancers such as breast, lung and renal cancer which generally form osteolytic metastases, with increased bone resorption. But this classification is probably oversimplified as both osteoclastic and osteoblastic activity might play a role in establishment and growth of prostate cancer metastases [22, 23]. The increased bone formation does not lead to a mechanically competent bone confirmed by prostate cancer patients often being prone to suffer from pathological fractures [24].

The way in which prostate cancer metastases affect bone remodeling can be described as a vicious cycle (Figure 1). Prostate cancer cells initially attach to the bone surface where they occupy a site normally taken by hematopoietic stem cells and adjacent to osteoblasts. Prostate cancer cells secrete osteogenic growth factors, such as ET-1, PDGF, BMPs, TGF-βs and IGFs, activating osteoblasts to form new bone matrix. The activated osteoblasts, in turn, secrete additional growth factors, including IGFs, FGFs and TGF-βs, which stimulate prostate cancer cell growth and proliferation. Tumor-derived growth factors and osteoblasts secreting receptor activator of the nuclear factor-κB ligand (RANKL) can also lead to activation of osteoclasts. The resulting bone resorption might
enhance the vicious cycle by creating more space for the osteoblastic lesion and by releasing cytokines from the bone matrix which further stimulates prostate cancer cells and osteoblasts [25, 26]

![Diagram of the vicious cycle of prostate cancer bone metastasis](image)

**Figure 1:** The vicious cycle of prostate cancer bone metastasis. Osteoblasts are stimulated by growth factors secreted by prostate cancer cells (e.g. ET-1, PDGF, BMPs, TGF-βs and IGFs). Activated osteoblasts (marker genes: ALPL, BGLAP and RUNX2) secrete additional growth factors (e.g. IGFs, FGFs and TGF-βs) that stimulates prostate cancer cells. Prostate cancer cells and osteoblasts stimulates osteoclasts (maker genes: ACP5, CTSK and MMP9) by trigging RANKL leading to prostate cancer cells being further stimulated by growth factors secreted from osteoclasts.

**Treatment and prognosis**
Localized prostate cancer is subjected to local treatment, radical prostatectomy or radiotherapy, and the intention is to cure the disease. Patients diagnosed at an early stage with a low risk tumor can be put under active monitoring or watchful waiting depending of their life expectancy. During active monitoring, the patient is put under surveillance and treatment is initiated at signs of tumor progression or at the patient's request. Watchful waiting can be used when the life expectancy is short and the main difference from active surveillance is that treatment is not initiated until signs of metastasis occur or there is a need to control symptoms. Patients diagnosed with locally advanced prostate cancer can be treated with local treatment or with androgen deprivation therapy (ADT) depending on the specific case (National Prostate Cancer Care Program, Sweden). Radiotherapy combined with ADT have been shown to improve survival compared to radiotherapy or ADT alone while a potential benefit of a combination of radical prostatectomy and ADT
have still not been shown [27-31]. ADT alone is a viable option if the patient is unfit or unwilling to go through curative therapy [32]. ADT is the first-line of therapy for patients with metastatic disease, i.e. advanced prostate cancer, and patients who progress following local treatment, which approximately one third of patients subjected to local treatment do (The National Board of Health and Welfare, Sweden).

The prognosis depends a lot on the extent of the tumor and its aggressiveness, only 28% of patients with advanced prostate cancer are alive after 5 years in comparison to patients with localized disease that has a 5-year survival of almost 100% and a 10-year survival of 98% [33].

Androgen deprivation therapy
In 1941, Charles Huggins and Clarence Hodges demonstrated that reduction of circulating androgens, through castration or estrogenic injections, had a suppressing effect on advanced prostate cancer reducing both symptoms and metastatic growth. Their findings, of which Huggins was rewarded with a Nobel Prize in 1966, lead to that ADT have become the gold standard for advanced prostate cancer therapy [34]. Patients may undergo either surgical (orchiectomy) or medical castration, both approaches have been shown to be equally effective in reducing tumor growth [35]. Medical castration is usually achieved through the use of gonadotropin-releasing hormone (GnRH)-agonists and -antagonists, the nomenclature of these drugs varies and there are several different to choose from but they act in a similar manner by targeting the release of luteinizing hormone (LH) from the pituitary gland leading to a decreased testosterone production. The main difference is that treatment using agonists results in an transient increase in testosterone, known as “testosterone flare”, and might cause unpleasant side effects for the patients before testosterone production eventually shuts down while the antagonists lowers testosterone without the initial increase [36]. In order to also block the androgens produced in the adrenal cortex, castration can be combined with anti-androgen drugs, such as the AR-antagonist bicalutamide. Although this combined androgen blockade (CAB) might reduce symptoms caused by “testosterone flare” the impact on survival is uncertain [37]. Bicalutamide monotherapy is an option for patients whom do not want to undergo or are unfit for surgical or medical castration, but it gives a slightly shorter median survival compared to castration [38]. Recent studies have also shown that a combination of ADT and chemotherapy, in the form of docetaxel, improves survival in patients with advanced prostate cancer [39]. Also, addition of the steroidogenesis inhibitor abiraterone acetate to ADT has been shown to prolong overall survival and progression-free survival compared to ADT alone [40, 41].
ADT has a negative impact on bone mineral density (BMD) leading to increased risk of osteoporosis and skeletal related-events (SREs) [42]. Therefore, ADT is often combined with bisphosphonates, which acts by inhibiting osteoclasts and osteolysis, and has been shown to decrease the risk of SREs [43]. However, bisphosphonates have not been shown to increase survival [39].

**Androgen receptor signaling**

Normal prostate function and development is controlled by androgens which binds to the androgen receptor (AR). In the adult prostate, the AR is expressed in both the epithelial and stromal cells [44]. AR signaling also plays a crucial role in prostate cancer [45].

The AR gene is located on the X chromosome (Xq11-12) and is a member of the nuclear steroid receptor superfamily of transcription factors. Similar to the modular structure of other steroid hormone receptors within this family, the AR gene is composed of eight exons encoding for a 110 kDa phosphoprotein with functionally distinct domains (Figure 2). The full-length AR protein structure consists of a NH2 terminal transactivation domain (NTD), a DNA binding domain (DBD), a hinge region containing the nuclear localization signal and the COOH terminal domain (CTD) which, due to its ligand-binding function, often is referred to as the ligand-binding domain (LBD) [46].

\[Figure 2: \text{Structure of the AR gene, transcripts and proteins (AR full-length, AR variant 7 and AR variant 576es). NTD: NH}_2\text{ terminal domain. DBD: DNA binding domain. H: hinge region. LBD: ligand binding domain. CE3: cryptic exon 3.}\]
Testosterone, the main circulating androgen, is synthesized by Leydig cells in the testes. Production of testosterone is regulated by the hypothalamus which secretes GnRH that binds to GnRH-receptors on gonadotropin cells in the pituitary gland thereby stimulating it to release LH. GnRH is also commonly referred to as LHRH (Luteinizing hormone-releasing hormone). LH stimulates the Leydig cells to produce testosterone. Testosterone production is regulated by a negative feedback loop and rising testosterone levels will inhibit release of GnRH from the hypothalamus (Figure 3). There is also a smaller amount of androgens produced by the adrenal cortex, such as dehydroepiandrosterone (DHEA) and androstenedione (4-dione), which can be converted into testosterone [47]. Although testosterone itself can bind to and activate the AR it is usually converted into dihydrotestosterone (DHT), a more potent androgen, within the prostate by 5α-reductase [48].

Figure 3: Testosterone regulation by the hypothalamic-pituitary-testicular axis. The hypothalamus secretes GnRH stimulating the pituitary gland to secrete LH which in turn stimulate testosterone by the testes. Testosterone production is regulated by a negative feedback loop.

In its inactive state, the AR is located in the cytoplasm in complex with chaperone proteins from the heat shock protein (HSP) family (Figure 4). When DHT, or another androgen, binds to the AR a conformational change occurs and the chaperones dissociate while the AR is translocated into the nucleus. In the nucleus the AR can control transcription by interacting with co-regulatory proteins and binding as a dimer to androgen response elements (AREs), specific DNA sites in the promoter and enhancer regions of androgen regulated genes such as KLK2, KLK3 (encoding for PSA), NXX3.1, STEAP2 and TMPRSS2 [49].
Figure 4: Androgen receptor signaling in prostate cancer. When inactive, The AR (full-length androgen receptor) is bound to HSPs (Heat-shock proteins) in the cytoplasm. Upon activation by DHT, or to a lesser extent testosterone, the AR is translocated into the nucleus where it forms a dimer, recruits co-regulators (e.g. FOXA1 and HOXB13) and binds to AREs (androgen response elements) in AR target genes (e.g. KLK2, KLK3, NKX3-1, STEAP2 and TMPRSS2) to initiate transcription of these genes leading to downstream effects which promotes prostate epithelial cell growth, survival and differentiation. Constitutively active AR-Vs (LBD-truncated AR variants, e.g. AR-V7 and AR-V567es) do not need to be activated by androgens in order to translocate to the nucleus. AR-Vs can form a homodimer, or a heterodimer together with the full-length AR, to recruit co-regulators and initiate transcription of target genes.
Castration-resistant prostate cancer
Initially ADT is a very effective treatment that decreases bone pain and lowers serum PSA levels in ~80-90% of patients with advanced prostate cancer. However, despite the initial remission of the disease sooner or later a relapse will occur. At this lethal end-stage the expected survival is only ~16-18 months. Although the time until relapse varies, the median is no longer than 2-3 years and only a small percentage of patients is still alive 10 years after initiating ADT [50, 51]. Because the disease progresses despite patients still have circulating androgens at a castration level this state is usually referred to as castration resistant prostate cancer (CRPC), other commonly used terms are androgen-independent prostate cancer or hormone-refractory prostate cancer [52]. However, the term “castration-resistant” is thought to more properly describe this state of disease since AR signaling still seems to play an important role in tumor progression after ADT and expression and activation of the AR is involved in most CRPC cases [53, 54]. There are several suggested mechanisms behind CRPC growth including, but not limited to, AR amplification and overexpression, AR mutations, AR splice variants, intra-tumoral androgen synthesis, abnormal activities of AR co-regulators, up-regulation of alternative signaling pathways and neuroendocrine differentiation [55, 56]. These mechanisms are summarized in a later section.

Treatment of castration-resistant prostate cancer
Because AR signaling still play an important role in prostate cancer progressing to CRPC, ADT is usually continued in these patients, sometimes switched to another agent or surgical castration and/or in combination with an anti-androgen such as bicalutamide (secondary CAB), or more recently with abiraterone or enzalutamide (see below). In CRPC without clinical evidence for metastases, secondary CAB have been shown to decrease patient serum PSA which is believed to prolong the time until metastases occur [57]. For metastatic CRPC, the first line treatment is usually chemotherapy in the form of docetaxel or novel anti-androgen therapies depending on whether the patient were treated with chemotherapy or abiraterone in the castration sensitive stage.

Docetaxel is a chemotherapeutic agent belonging to the taxoid family of drugs, whose members are usually referred to as taxanes. Taxanes inhibits cell division by disrupting the normal function of microtubules [58]. In 2004, two large randomized phase III trials reported a benefit in overall survival when treating patients suffering from metastatic CRPC with docetaxel versus mitoxantrone, the standard treatment against CRPC at the time. In fact, docetaxel was the first therapy showing an increased survival in CRPC patients, previous treatments could only show palliative benefits, and based upon these results docetaxel has been the standard treatment for CRPC for many years. [59-61].
In recent years there has been an extensive development and several new drugs, many based on different mechanisms of action, have been shown to prolong survival and increase quality of life for CRPC patients. Some of these treatments, which are summarized next, have now been approved and are available for clinical use. [62]

**Cabazitaxel** (Jevtana®) was the first drug shown to improve survival in patients with metastatic CRPC progressing after docetaxel treatment. It is a second-generation taxane that was selected for clinical development by screening a large set of taxane derivatives based on their microtubule stabilizing activity in taxane resistant cell lines and a docetaxel-resistant *in vivo* tumor-model. [63-65]

**Abiraterone acetate** (Zytiga®) is a steroidogenesis inhibitor that acts by inhibiting CYP17A1, an important enzyme for androgen biosynthesis, thereby blocking steroid production by the adrenal glands and testes and it also blocks intratumoral steroid production. Abiraterone has been shown to provide survival benefits to both CRPC patients who have progressed on docetaxel and those who have not received any prior chemotherapy [66, 67].

**Enzalutamide** (Xtandi®) is a novel AR antagonist that has shown significantly prolonged survival of men with metastatic CRPC when given before as well as after chemotherapy. Enzalutamide has a very high affinity for the AR and it acts by targeting multiple steps in the AR signaling pathway; preventing androgen binding, AR nuclear translocation and AR binding to DNA. Similar to bicalutamide, enzalutamide binds to the LBD of the AR [68, 69].

**Radium-223 dichloride** (Xofigo®) is an alpha emitter, a radioisotope therapy that targets bone metastases by selectively binding to areas with an increased bone turnover. It will bind into the newly formed bone stroma where the short-ranged alpha radiation will produce a localized cytotoxic effect by inducing double-stranded DNA breaks in the surrounding cells. Radium-223 has been shown to improve overall survival in CRPC patients with bone metastases regardless of whether they had received previous chemotherapy or not [70, 71].

**Sipuleucel-T** (Provenge®), a cell-based immunotherapy, prolongs survival in patients with metastatic CRPC by using the patient’s own immune system. Immune cells from the patient are activated *ex vivo* with a recombinant fusion protein consisting of a prostate antigen, prostatic acid phosphatase (PAP), fused to an immune cell-activator, GM-CSF. When the immune cells are reinfused into the patient they will induce an immune response towards prostate cancer cells [72]. Sipuleucel-T is currently not approved for use outside the U.S.
**Mechanisms behind castration-resistant prostate cancer**

**AR amplification and overexpression**

Despite ADT, some androgens still exist at the CRPC stage. A common way for CRPC tumors to adapt to the low levels of androgens is to become hypersensitive to androgens by AR gain or amplification. Up to 80% of CRPC cases have an elevated AR gene copy number and 20–60% have AR amplification. Notably, very few hormone-sensitive (hormone-naïve) prostate cancers carry AR amplifications indicating that this aberration is selected for during the development of CRPC [73-79]. *In vitro* studies has shown that cell lines overexpressing the AR through AR amplifications make the AR hypersensitive, androgen-regulated genes are up-regulated by 10-fold lower concentration of DHT than in control cells, and chromatin binding of the AR take place in 100-fold lower ligand concentration [80, 81]. Interestingly, recent studies have shown that AR amplification is more common in CRPC patients progressing after enzalutamide treatment than patients who had received abiraterone or other agents indicating that AR amplification is a possible resistance mechanism towards enzalutamide while abiraterone treatment might select for cancer cells without AR amplification [78, 79].

**AR mutations**

Although rare in untreated early stage prostate cancer AR mutations are more common in CRPC and is present in up to 30% of cases. The highest frequencies are seen in CRPC treated with anti-androgens while more uncommonly seen in CRPC treated by castration alone [82]. Most mutations occur in the LBD enabling a promiscuous activation of the AR by weak adrenal androgens and non-androgenic steroids such as DHEA, estrogen, progesterone and glucocorticoids. [83-87]. But the mutations can also be activated by AR-antagonists, such as flutamide and bicalutamide, thereby making them function as agonists instead [88, 89]. Abiraterone treatment has been shown to select for progesterone responsive AR mutants because CYP17A1 inhibition leads to a cellular increase of progesterone [90]. AR mutations have also been shown to give resistance to enzalutamide and other novel anti-androgens [91].

**AR variants**

Constitutively active AR variants (AR-Vs) were first described in a paper from 2002 by Tepper et al. who showed the occurrence of a ~112 kDa and a ~75-80 kDa AR mutant in 22Rv1 cells, a cell line derived from a CWR22 xenograft which relapsed during ADT. The smaller ~75-80 kDa AR protein was found to lack the C-terminal LBD [92]. Further characterization of the truncated AR protein showed that it maintained its transcriptional ability despite the lack of LBD and it was first suggested that proteolytic cleavage of the full-length AR was responsible for this truncated isoform [93]. Nowadays, alternative splicing due to
incorporation of cryptic exons in the AR gene is considered to be the underlying cause to many of these variants but some may also be caused by exon skipping or by genetic deletions and rearrangements [94]. Studies have shown that siRNA targeting the LBD only reduce the expression of full-length AR and not AR isoforms lacking the LBD suggesting that truncated AR variants are not products of the full-length AR but instead are derived from unique RNAs [95]. Today, more than 20 different AR splice variants has been identified although in some cases more than one described variant might actually refer to the same isoform because several investigators have been involved, which has led to a varied nomenclature. Among these variants only a few have been shown to be constitutively active while others are either believed to be conditionally active or their function is more unclear [94, 96, 97].

The two most well studied constitutively active AR variants are AR-V7 (also referred to as AR3), discovered in the 22Rv1 and CWR-R1 cell lines, and AR-V567es that was originally found in the LuCaP xenografts 86.2 and 136 (Figure 2 and 4) [98-100]. An early study from our group showed that both these AR variants are expressed in bone metastases from CRPC patients and that high levels of AR-V7 and/or detection of AR-V567es was associated with a particularly poor prognosis. Moreover, high levels of AR-V7 transcripts was strongly correlated to expression of LBD-truncated AR protein [101]. Notably, high levels of AR-V7 was also associated with AR amplification [77]. Since most AR-targeting therapies relies on a receptor with an intact LBD it has been suggested that CRPC expressing constitutively AR variants lacking the LBD will have a poor response to these treatments, including newer agents such as enzalutamide and abiraterone. [102]. Indeed, studies have shown that CRPC patients with detectable levels of AR-V7 in circulating tumor cells (CTCs) or in peripheral blood are likely to show resistance to enzalutamide and abiraterone [103-106]. Instead, it has been suggested that taxanes, such as docetaxel and cabazitaxel, might be a more suitable treatment for CRPC patients expressing AR-V7 [106-108].

Intra-tumoral androgen synthesis
Even though circulating androgens are at castrate-levels it has been shown that intra-tumoral androgen levels in CRPC often are the same as or higher than in eugonadal men [109]. It is believed this is due to conversion of adrenal steroids, such as DHEA and androstenedione, to testosterone and DHT rather than de novo steroidogenesis [110-112]. A study from our group showed increased expression of some steroidogenic enzymes, AKR1C3 and SRD5A1, in sub-group of CRPC bone metastases. AKR1C3 and SRD5A1 are enzymes involved in the conversion of DHEA and androstenedione into testosterone and DHT, and because the enzymes needed to convert cholesterol into DHEA and androstenedione, CYP11A1, CYP17A1 and HSD3B2, were expressed at lower levels than in non-malignant prostate tissue we concluded that it is more likely that
adrenal androgens than *de novo* androgen synthesis from cholesterol contribute to CRPC growth. Another interesting discovery was that high protein expression of AKR1C3 in most cases did not coincide with expression of AR-Vs indicating that these two mechanisms probably develops separately from each other [113]. Studies on prostate cancer cell lines and xenografts have indicated that up-regulation of enzymes within the steroidogenesis pathway, including CYP17A1 and AKR1C3, might be a potential resistance mechanism against abiraterone treatment [114, 115].

**Aberrant activation by alternative pathways**

Up-regulation of other signaling pathways have been shown to promote cell survival and proliferation in CRPC including NF-κB, PI3K/AKT and Glucocorticoid receptor (GR) [55, 56]. The NF-κB pathway, which is involved in many different cancers, has been shown to contribute to the development of resistance towards enzalutamide in prostate cancer cell lines by inducing expression of AR-Vs and activation of AR during androgen-depleted conditions [116]. Activation of the PI3K/AKT signaling pathway via loss of the tumor suppressor PTEN is commonly seen in metastatic prostate cancer and has been associated with CRPC growth [117]. It has been suggested that the AR and PI3K/AKT pathways cross-regulate each other by reciprocal feedback; inhibition of one pathway will activate the other [118]. This reciprocal feedback indicates that up-regulation of PI3K/AKT signaling is a potential resistance mechanism towards AR inhibition and studies on preclinical CRPC models have shown that a combined inhibition of AR and PI3K/AKT prolong disease stabilization [119]. Also, a phase II study combining abiraterone treatment with an AKT inhibitor, Ipatasertib (GDC-0068), in patients with metastatic CRPC concluded that the combination therapy was superior over abiraterone alone, especially in patients whose tumors showed loss of PTEN [120].

Glucocorticoids are often used in clinical practice, including prostate cancer treatment, because of various reasons such as anti-inflammatory effects and their ability to mitigate pain but also because they have some anti-tumor effects and can suppress adrenal androgen synthesis. Glucocorticoids are usually given together with chemotherapies, such as docetaxel and cabazitaxel, to counteract the side effects and provide palliative benefits. They are also added to abiraterone treatment because inhibition of CYP17A1 leads to a reduction of glucocorticoids causing a compensatory production of mineralcorticoids, not hindered by CYP17A inhibition, leading to side effects such as hypokalemia and hypertension. Addition of a synthetic glucocorticoid, such as prednisone, decreases the levels of these mineralcorticoids via negative feedback. [121]. However, the GR, belonging to the same steroid receptor superfamily as the AR, has been shown to be able to bind to many AR regulated genes suggesting that signaling via the GR might be a way for CRPC to develop resistance toward AR targeting agents [122].
Abnormal activities of AR co-regulators
There are several different molecules identified as co-regulatory proteins of the AR. Co-activators enhance transcription of AR regulated genes while co-repressors suppress the transcriptional activity. Many co-activators are enzymes which modulates other proteins within the co-regulatory complex through phosphorylation, methylation, acetylation or ubiquitylation but there are also proteins with numerous other functions such as chaperones and proteins involved in RNA metabolism and splicing [123]. Many co-activators have been suggested to contribute in prostate cancer progression such as FKBP51, the SRC family (SRC-1/NCOA1, SRC-2/GRIP-1/TIF-2/NCOA2 and SRC-3/AIB1/NCOA3) and p300. Accordingly, co-repressors might be reduced in CRPC. [56].

Neuroendocrine differentiation
Although the vast majority of prostate cancers are classified as adenocarcinomas, originating from the epithelial cells of the prostate gland, a rare subset of tumors originates from neuroendocrine cells. While their function is still quite unclear, neuroendocrine cells are believed to be involved in the regulation of prostate growth and differentiation and in regulating the secretory processes of the prostate gland. Neuroendocrine cells are considered androgen independent, they do not express the AR or PSA, which means that neuroendocrine prostate cancer (NEPC) naturally do not respond well to ADT and have a generally poor prognosis [124, 125]. Primary NEPC is very rare and there are several different types of NEPC proposed such as small cell carcinoma (SCC) of the prostate and large cell neuroendocrine carcinoma (LCNEC) but also NEPC mixed with adenocarcinoma [126, 127]. Because neuroendocrine cells possess intrinsic androgen independence, neuroendocrine differentiation (NED) is a proposed mechanism for developing CRPC. NED is more frequently observed in CRPC than in hormone sensitive prostate cancer and has been associated with loss of AR indicating resistance towards therapies targeting AR and AR signaling [128-130].

Epigenetic dysregulation
Epigenetic alterations, such as tumor suppressors being silenced by hypermethylation, have long been reported to occur in prostate cancer and epigenetic changes have also been suggested as a plausible mechanism driving CRPC progression. Alterations in epigenetic master regulators have been proposed to enhance transcriptional activity of AR signaling and also to activate other oncogenic signaling pathways contributing to aggressiveness and androgen independence. [131, 132] Epigenetic changes is also suggested to contribute to NED because loss of AR have been associated with hypermethylation of the AR promoter region in CRPC [133]. Also, AR negative preclinical prostate cancer models treated with the demethylating agent 5-Azacitine has been shown to restore AR expression thus improving the anti-tumor effect of bicalutamide [134, 135].
Aims

Overall aim
Patients with metastatic CRPC have very poor prognosis and novel treatments are constantly under development, many of which are now being available in the clinic. Due to heterogeneous molecular mechanisms underlying CRPC development and newly developed therapies having different mechanisms of action, CRPC patients will likely show diverse therapy responses. Today, no biomarkers are used for patient stratification into different treatments.

The overall aim of this thesis was to characterize bone metastases and CRPC in patients and preclinical models in order to identify molecular heterogeneities of biological/pathological relevance that could be used to predict therapy response/resistance and aid in the development of novel treatment strategies for metastatic disease.

Specific aims

Paper I
To characterize the gene expression pattern of bone metastases from men with CRPC, in order to identify subgroups of relevance for therapy choice.

Paper II
To investigate bone cell activity in clinical prostate cancer bone metastases in relation to tumor cell AR activity, in order to gain novel insight into biological heterogeneities of possible importance for patient stratification into bone-targeting therapies.

Paper III
To explore the DNA methylation pattern of clinical prostate cancer bone metastases in relation to molecular heterogeneity observed at the RNA expression level, as well as in relation to the general methylation pattern of normal prostate tissue and primary prostate tumors.

Paper IV
To investigate the effects of abiraterone and cabazitaxel, and subsequently developed resistance mechanisms, in 22Rv1 prostate cancer xenografts expressing high levels of constitutively active AR variants.
**Materials and methods**

This section contains a summary of the materials and methods used in the thesis, for a more detailed description, see the corresponding papers.

**Patient materials (paper I-III)**

Patient bone metastasis samples were obtained from a series of biopsies collected from men with prostate cancer or other malignancies who underwent surgery for metastatic spinal cord compression at Umeå University Hospital (2003–2013). Matched diagnostic prostate biopsies were available in some cases.

Tissue samples of non-malignant prostate and prostate cancer from 13 separate men who were treated with radical prostatectomy at Umeå University Hospital between 2008 and 2009 was also included.

Tissue microarrays (TMAs) were previously constructed from samples obtained from patients undergoing transurethral resection of the prostate (TURP) at the Central Hospital in Västerås between 1975 and 1991. For a more detailed description see [136].

All patients gave their informed consent, written or verbal, for inclusion before participation and the study was approved by the local ethic review board of Umeå University.

**Tissue preparation (paper I-III)**

Bone metastasis samples were instantly fresh-frozen in liquid nitrogen or fixed in 4% buffered formalin. Fixed samples were decalcified in 20% formic acid at 37°C for 1-3 days, depending on the sample size, followed by paraffin-embedment.

Immediately after radical prostatectomy, prostate samples were brought to the pathology department, cut in 0.5 cm thick slices before 20 samples were taken from each prostate using a 0.5 cm skin punch and frozen in liquid nitrogen within 30 minutes after surgery. The remaining prostate slices were formalin-fixed, paraffin-embedded and whole-mounted as 5 µm thick sections before hematoxylin-eosin staining. The composition of the frozen samples, non-malignant or malignant prostate tissue, was determined by a histological evaluation of their location in the whole-mount section but also verified in hematoxylin/eosin-stained sections from each frozen sample. Both malignant and non-malignant tissue samples were included for each patient.
**Extraction of RNA, DNA and Protein (paper I-IV)**

Frozen tissue samples were cryo-sectioned into extraction tubes containing lysis buffer. Parallel sections were mounted on glass slides and stained with hematoxylin-eosin followed by a histological examination of the sample tissue composition, such as bone tissue and tumor cell content. Cell culture samples were trypsinized, spun down to pellets and washed in PBS before addition of lysis buffer. Isolation of RNA, DNA and protein was performed by using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen), according to manufacturer’s protocol and the protein fraction was dissolved in 5% SDS. In some cases RNA was isolated using the TRIzol protocol (Invitrogen). RNA and protein concentrations were quantified by absorbance measurements using a spectrophotometer (ND-1000; NanoDrop Technologies or DeNovix DS-11 FX+ microvolume spectrophotometer; AH Diagnostics). RNA quality was analyzed with a 2100 Bioanalyzer (Agilent Technologies). DNA quality and quantity was determined by spectrophotometry (ND-1000; NanoDrop Technologies) and the Qubit dsDNA BR assay kit on a Qubit 3.0 Fluorometer (Invitrogen). For specific details regarding sample inclusion criteria, see paper I-IV.

**RNA analysis**

**Quantitative real-time RT-PCR (paper I)**

Total RNA was reversed transcribed into cDNA using Superscript VILO (Thermo Fisher Scientific). Quantification of mRNA levels was performed using TaqMan assays for HLA-A (Hs01058806_g1), TAP1 (Hs00388675_m1), and PSMB9 (Hs00160610_m1) on an ABI Prism 7900HT Sequence Detection System according to the manufacturers’ protocols (Thermo Fisher Scientific). Each sample was run in duplicates and adjusted for the corresponding RPL13A mRNA level (Hs01578912_m1, Thermo Fisher Scientific) using the ddCt method.

**Whole-genome expression arrays (paper I-IV)**

Amplification of total RNA was made with the Illumina TotalPrep RNA amplification kit (Ambion) and the generated cRNA was hybridized to HumanHT-12 Expression BeadChips (Illumina) according to the manufacturers’ protocols. Beadchips were scanned using a HiScan system (Illumina) and array data was processed and normalized using the GenomeStudio software (version 2011.1, Illumina). Probes with all signals lower than two times the mean background level were excluded.
DNA analysis

**DNA methylation profiling (paper III)**
Genomic DNA was bisulfite converted using the EZ DNA Methylation Kit (Zymo Research) and thereafter applied to the Infinium Methylation EPIC arrays (Illumina), and operated according to the manufacturer’s instructions.

Array analysis including pre-processing and normalization was performed as previously described [137], with some modifications. The quality of each individual array was evaluated with built-in controls and the matching identities of the non-malignant prostate and primary prostate cancer paired samples was confirmed by using the 59 built-in SNPs. The fluorescence intensities were extracted using the Methylation Module (1.9.0) in the GenomeStudio software (V2011.1, Illumina), whereas pre-processing and downstream analysis was done using R (v3.4.1). Data was normalized using the BIMQ method to compensate for the two different bead types used in the array [138]. CpG probes that align to multiple loci in the genome or were located in methylation quantitative trait loci (meQTLs) [139, 140] or located less than 5 bp from a known single nucleotide polymorphism in the European population [141] were excluded. CpG probes with detection p-value > 0.05 in any sample were also excluded. The methylation level (β-value) of each CpG site ranging from 0 (no methylation) to 1 (complete methylation) was used as measure for methylation level in down-stream analyses. Methylation levels (β-values) were extracted for promoter associated CpGs located in the TSS1500, TSS200, and 5’UTR regions, which showed an overall SD > 0.05. Differentially methylated CpG sites were defined as a mean delta-β-value > 0.3 or < - 0.3 between compared groups. The CpG sites were matched to gene transcripts by their Entrez gene identification number.

Protein analysis

**Western blot (paper IV)**
Samples were separated by 10% SDS-PAGE gels or 4-20% Mini-PROTEAN TGX stain-free protein gels (Bio-Rad Laboratories) before transferred to nitrocellulose membranes using the Trans-Blot Turbo transfer system (Bio-Rad Laboratories). Membranes were blocked in LI-COR blocking buffer (LI-COR Biosciences) followed by incubation primary antibodies targeting ABCB1 (C219; BioLegend), AR (N-20; Santa Cruz Biotechnology), Nkx3.1 (N6036; Sigma-Aldrich) and β-actin (A5441, Sigma-Aldrich). Protein expression was visualized using LI-COR Odyssey fluorescently labeled IRDye 800CW and IRDye 680RD secondary antibodies and analyzed using a LI-COR Odyssey CLx scanner and the ImageStudio 3.1.4 software (LI-COR Biosciences).
**Immunohistochemistry (paper I-IV)**

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated through graded ethanol. For histological examinations, sections were stained with hematoxylin-eosin and/or van Gieson solution. Immunohistochemistry was performed using primary antibodies for ABCB1 (C219; BioLegend), AR (MUC256-UCE; Biogenex or N-20; Santa Cruz Biotechnology), AR-V7 (31-1109-00; RevMAb Biosciences), BMP4 (ab39973; Abcam), CD3 (NCL-L-CD3-565; Novocastra), CD68 (M0814; Dako), FOXA1 (ab23738; Abcam), HLA class I ABC (ab70328; Abcam), PSA (A0562, Dako), RUNX2 (ab81357; Abcam) and TRAP (MABF96; Millipore). For more detailed descriptions of the morphological and immunohistochemical evaluations, see papers I-IV.

**Cell culturing and xenograft experiments**

**Cell line (paper IV)**

The 22Rv1 cell line (ATCC, CRL-2505) was maintained according to ATCC instructions in RPMI 1640+GlutaMAX supplemented with 10% fetal bovine serum (FBS) or 10% charcoal-stripped FBS, 100 U penicillin/mL and 100 µg streptomycin/mL, 10 mM HEPES and 1 mM Sodium Pyruvate (Thermo Fisher Scientific).

**Nude mice xenografts (paper IV)**

22Rv1 cells were diluted 1:1 in RPMI (Thermo Fisher Scientific) and Matrigel (BD biosciences) before injected subcutaneously into the flanks of 8-weeks-old, athymic male BALB/c nude mice (Scanbur). Tumor volume was measured 2-3 times per week by calipers and calculated by length x (width^2)/2. When tumors reached approximately 100 to 200 mm^3 mice were randomly selected for treatment with castration by surgical incision, abiraterone or cabazitaxel. Treatment with abiraterone and cabazitaxel was given either as monotherapy or in combination with castration. To select for resistance, a subset of animals treated with castration plus cabazitaxel received repeated cabazitaxel treatment at tumor regrowth. Control animals received sham operation, vehicle for abiraterone or cabazitaxel with or without sham operation.

Abiraterone acetate (kindly provided by Janssen Pharmaceutica) was diluted to 40mg/mL in 5% benzyl alcohol, 95% safflower oil and given daily by i.p. injections of 0.5 mmol/kg. Cabazitaxel was received as frozen aliquots of Jevtana® (Sanofi) 10mg/mL, 24% polysorbate 80, 9,8% EtOH stock solution (left-overs from patient treatments at the Oncology clinic, Umeå University Hospital) and diluted to 2.083mg/mL in 5% polysorbate 80, 5% glucose and 2%
EtOH before given as 2 weekly injections of 20 mg/kg. Mice that showed a body weight loss <10% received a third injection.

The experiment was terminated when tumors reached a volume of about 1000 mm³. Tumors and prostate tissue were dissected and weighed before divided and flash frozen in liquid nitrogen or fixed in 4% paraformaldehyde. Animal work was carried out in accordance with protocol approved by the Umeå Ethical Committee for Animal Studies.

**Establishment of cabazitaxel-resistant cell lines (paper IV)**

Two different 22Rv1 xenografts relapsing during repeated cabazitaxel-treatment were established as cell lines; termed 22Rv1-CabR1 and 22Rv1-CabR2, as further described. Tumor tissue was aseptically minced using scissors and dissolved by 0.1% collagenase (Sigma-Aldrich) in Hanks' Balanced Salt Solution (HBSS) containing calcium and magnesium (Thermo Fisher Scientific) while incubated at 37°C for 1h. After incubation, cells were filtered through a 100µm cell strainer and washed with HBSS free from calcium and magnesium. Filtered cells were centrifuged twice, resuspended in growth media (RPMI with 10% FBS, Thermo Fisher Scientific) and seeded into cell culture flasks. When the cells had become established and showed stable growth the media was changed to RPMI with 10% charcoal stripped FBS (Thermo Fisher Scientific). For further selection the cells were cultured in media containing cabazitaxel gradually increasing at each passage up to a final concentration of 10 nmol/L. 22Rv1 cells grown in charcoal stripped media together with vehicle was used as control. Cells were grown without cabazitaxel or vehicle for 1-2 passages before experiments.

**Evaluation of cabazitaxel resistance in vitro (paper IV)**

Cabazitaxel-resistance was tested *in vitro* by growing cell samples as triplicates in 6-well plates for 9 days in media supplemented with cabazitaxel up to 100 nmol/L and counted using a Countess Automated Cell Counter (Thermo Fisher Scientific).

To examine if 0.25 µmol/L elacridar (Sigma-Aldrich), 20 µmol/L bicalutamide (Sigma-Aldrich) or 20 µmol/L enzalutamide (Selleckchem) could reverse the *in vitro* cabazitaxel resistance, the resistant cell lines were grown in quadruplicates of 10 000 cells in 96-well plates (IsoplateTC, Wallac, Finland) for 96 hours in media containing 0 to 10 nmol/L cabazitaxel ± each inhibitor. Cell viability was assayed with CellTiter Glo 2.0 according to the manufacturer’s instructions (Promega). Luminescence was measured using a SpectraMax i3x Multi-Mode Detection Platform (Molecular Devices).
Sensitivity towards simvastatin (Sigma-Aldrich) was measured as described above using 0.1 to 100 µmol/L. Simvastatin was activated according to the manufacturer’s instructions.

**Verification of cabazitaxel resistance in vivo (paper IV)**
The *in vivo* cabazitaxel-resistance was tested by establishing xenografts from 22Rv1-CabR1 and 22Rv1-CabR2, as described above, and treating them with castration and cabazitaxel. 22Rv1 was used for control xenografts. Cells were cultivated in charcoal-stripped media with cabazitaxel or vehicle as described above until 1-2 passages prior to injection when cabazitaxel or vehicle was removed. All mice were surgically castrated four days before injection of cancer cells and were treated with cabazitaxel, by 2 weekly injections of 20 mg/kg, when tumors reached the required size (100-200mm³). As before, when tumors reached a volume of ~1000mm³ the mice were sacrificed and ventral prostate (VP) and tumor tissue were collected and frozen/fixated.

**Statistical analysis**

**Univariate analysis (paper I-IV)**
Correlations between variables were investigated using the Spearman rank test. Groups were compared using the Kruskal-Wallis test, the Mann-Whitney U-test or the independent samples t-test for continuous variables and the χ² test for categorical variables. Paired samples were compared using the Wilcoxon signed-rank test. The Kaplan-Meier method was used for survival analysis, with death from prostate cancer as events and death from other causes as censored events. Univariate statistical analyses were performed using the latest version of SPSS software (SPSS Inc.). *P*≤0.05 was considered statistically significant.

**Multivariate data analysis (paper I-IV)**
Multivariate modelling using principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) were used to create an overview of the variations in whole-genome expression data. Multivariate statistical analyses were performed in SIMCA version 14.0 (Umetrics, Umeå, Sweden).

**Functional enrichment analysis (paper I-IV)**
Functional gene set enrichment analysis (GSEA) was performed using the Ingenuity Pathway Analysis (IPA; Qiagen) or the MetaCore software (Clarivate analytics).
Results and discussion

Paper I

Subgroups of castration-resistant prostate cancer bone metastases defined through an inverse relationship between androgen receptor activity and immune response

Patient response to androgen ablation and AR-targeted therapies varies, probably due to heterogeneous mechanisms behind castration-resistance, stressing that biomarkers for treatment stratification are needed. Specifically, CRPC by-passing the need for AR activity will probably not respond to any AR targeting therapy but need different treatment options. The aim of this study was to identify subgroups among clinical bone metastases which could be of relevance for when choosing between different therapies.

In this study, we characterized a set of fresh-frozen bone metastases from patients with CRPC (n=40) in comparison to bone metastases from patients with treatment-naïve prostate cancer (n=8) and bone metastases from untreated patients with other primary malignancies (n=12), using whole-genome expression profiling followed by multivariate data analysis and functional enrichment analysis.

Results from the multivariate PCA model showed that while the majority of the CRPC bone metastasis samples cluster close to untreated prostate cancer metastases, some CRPC metastases cluster closer to the metastases of other cancer origin. Based on the first score vector (t[1]), capturing the largest variation in the data, CRPC samples could be divided into two subgroups. We defined the larger subgroup (80% of CRPC samples) as AR-driven due to high expression levels of the AR, AR co-regulators FOXA1 and HOXB13, as well as androgen-regulated genes such as KLK2, KLK3, NKX3-1, STEAP2, and TMPRSS2. Accordingly, the smaller subgroup (20% of CRPC samples) showed lower levels of these gene transcripts and was defined as non-AR-driven. Also, serum PSA at the time of metastasis surgery was higher in patients with AR-driven CRPC metastases than patients with non–AR-driven CRPC metastases.

Functional differences between these two subgroups was analyzed by importing a list of differently expressed genes (fold-change ≥ ± 1.5, P≤00.1) into the IPA tool for assignment of altered canonical pathways and identification of upstream regulators. Analysis of upregulated canonical pathways showed that AR-driven CRPC samples have higher metabolic activity, such as cholesterol biosynthesis, fatty acid β-oxidation and polyamine synthesis, compared to non-AR-driven
CRPC. Further investigation showed that most pathways downregulated in AR-driven CRPC belong to cellular immune response, such as the antigen presentation pathway. AR, SPDEF and FOXA1 were found among top activated upstream regulators predicted as responsible and they also showed increased transcript levels in AR-driven samples, while several immune regulating genes and cytokines were predicted to be inhibited, of which some also showed reduced transcript levels. No difference in neuroendocrine and cancer stem cell markers were found between subgroups.

Based on these results, we describe for the first time two subgroups among CRPC bone metastases, defined by high AR activity and low cellular immune response, or low AR activity and high cellular immune response. Earlier studies within our group have shown increased expression of constitutively active AR variants or the steroidogenic enzyme AKR1C3 in subgroups of CRPC bone metastases [101, 113]. As expected, the AR-driven subgroup expressed much higher levels of AR-V7. But, no difference in AKR1C3 expression was seen between AR-driven and non-AR-driven CRPC bone metastases. The finding that AR-driven CRPC bone metastases show a higher metabolic activity is in line with previous studies by our group showing high cholesterol levels and β-oxidation in prostate cancer bone metastases [142, 143]. In addition, shortly after we published this current study we received a letter to the editor encouraging us to further investigate the upregulation of cholesterol biosynthesis and specifically the possible role of CYP27A1, an enzyme involved in regulation of cholesterol homeostasis shown to be downregulated during prostate cancer progression [144]. AR-driven metastases showed clearly lower CYP27A1 expression and higher expression of HMGCR, coding for the rate-limiting enzyme HMG-CoA reductase in cholesterol biosynthesis, compared to non-AR-driven metastases further strengthening our results indicating that cholesterol biosynthesis contributes to the development of AR-driven CRPC [145]. Therefore, we hypothesize that besides AR-targeting therapy, there might be a potential benefit of treating patients with AR-driven CRPC metastases with therapies targeting cholesterol biosynthesis, β-oxidation, and polyamine synthesis. However, patients with non-AR-driven CRPC metastases will probably be resistant to all form of AR-targeting therapies, but because they show higher cellular immune response, they might be susceptible to immunotherapy.

In cancer immunotherapy, various types of immunologic interventions are used to stimulate and improve the immune system’s innate antitumor response. Tumors can use different mechanisms for immune evasion. Tumor cells, regulatory T-cells (Tregs), and myeloid-derived suppressor cells (MDSCs) can express inhibitory factors that suppress cytotoxic CD8+ T-cells and NK cells and MDSCs and M2 macrophages can promote tumor growth by secreting factors stimulating angiogenesis and tumor cell invasion[146, 147].
Most cancer immunotherapies that have been tested clinically against prostate cancer fit into two general types; vaccine-based immunotherapies, with the goal to stimulate an antitumor response against prostate-specific or tumor-specific antigens, and inhibitors targeting immune checkpoint pathways. Vaccine-based therapies include sipuleucel-T; a FDA approved dendritic cell–based vaccine using PAP as antigen, PROSTVAC; a PSA-targeting viral-based vaccine, and GVAX; a whole-cell–based vaccine derived from two prostate cancer cell lines modified to secrete the immunostimulating cytokine GM-CSF [148]. Immune check point inhibitors include ipilimumab and pembrolizumab, two drugs successful in melanoma treatment and the researchers behind them was recently awarded with the Nobel Prize in Medicine 2018. Ipilimumab is a human monoclonal antibody inhibiting CTLA-4 (cytotoxic T-lymphocyte–associated antigen 4) an inhibitory receptor that downregulates T-cell activation pathways. Pembrolizumab is also a monoclonal antibody that acts by inhibiting PD-1 (programmed cell death protein 1), a receptor that suppress T-cell response by binding to PD-L1 (PD-1 ligand 1). Upregulation of PD-L1 has been shown to contribute to tumor immune evasion [146]. Tasquinimod is an immunotherapeutic drug that does not fit into either group, being a small-molecule inhibitor targeting S100A9, an immunoregulatory protein expressed on MDSCs [148].

The immune cell profile in prostate cancer metastases is largely unknown, but studies on primary prostate tumors have shown low tumor infiltration of T-cells, B-cells, and monocytes in advanced disease that was associated with poor prognosis [149]. However, tumor infiltration of specific subtypes of lymphocytes/monocytes, such as FoxP3+ Tregs, CD163+ immunosuppressive M2 macrophages, and S100A9-positive inflammatory cells, have been shown in lethal prostate cancer [150-154]. Also, high frequencies of Tregs and MDSCs in peripheral blood as well as whole-blood-based mRNA profiling mirroring high monocyte/low lymphocyte numbers have been related to poor prognosis in CRPC patients [155, 156].

Immunohistochemical analysis confirmed the difference in cellular immune response indicated between non–AR- and AR-driven at the transcriptomic level, by showing a significantly higher volume density of CD68+ monocytes and borderline higher frequency of CD3+ infiltrating T lymphocytes in FFPE samples in non–AR-cases. Furthermore, gene expression data showed that non-AR-driven samples have higher levels CD8+ effector T-cells and CD4+ helper T-cells, but also higher levels transcript coding for inhibitory T-cell receptors CTLA4 and PDCD1 (coding for PD-1) as well as decreased levels of TBX21, a proinflammatory Th1 transcription factor while transcript levels of the stimulatory T-cell receptors ICOS and CD28 and the anti-inflammatory Th2 transcription factor GATA showed not difference between subgroups. FOXP3, a Treg transcription factor,
could not be detected. High levels of \textit{CD163}, and \textit{S100A9} but not the proinflammatory M1 macrophage marker \textit{NOS2}, indicated metastasis infiltration of tumor-promoting M2 macrophages and MDSCs.

Because the lower immune cell infiltration seen in AR-driven CRPC metastases possibly could be caused by downregulation of MHC class I antigen presentation we choose to further investigate expression of genes involved in this pathway, \textit{PSMB9}, \textit{TAP1}, and \textit{HLA-A}, using qRT-PCR in an extended set of bone metastases; 53 CRPC, 11 treatment-naive prostate cancer and 13 untreated metastases from other malignancies. Also, 12 paired samples of non-malignant and malignant prostate tissue from radical prostatectomies was used for comparison. Analysis of \textit{PSMB9}, \textit{TAP1}, and \textit{HLA-A} mRNA expression showed lower levels in prostate cancer samples than samples from metastases of other cancer origin but also that CRPC samples could be separated into two groups based on expression levels of these genes indicating that while downregulated in most CRPC bone metastases, MHC class I antigen presentation is intact within a subgroup of CRPC. Expression of \textit{PSMB9}, \textit{TAP1}, and \textit{HLA-A} was significantly lower in malignant prostate tissue compared to nonmalignant prostate tissue and prostate cancer bone metastases showed the lowest levels indicating a reduction of MHC class I antigen presentation during prostate cancer disease progression. Immunostaining of HLA class I ABC confirmed these findings by showing lower immunoreactivity in metastases samples than in their matched primary tumor biopsies taken at diagnosis before metastasis surgery. While previous work have reported downregulation of MHC class I in docetaxel-resistant prostate cancer cells [157], we did not find any enrichment of MHC class I negative tumors among patients treated with docetaxel.

Based on gene expression data, the multivariate PCA model suggested an inverse correlation between AR-activity and immune response such as the antigen presentation pathway. To further investigate this relation we compared HLA class I ABC immunoreactivity in relation to nuclear AR immunoreactivity. We have previously measured AR immunoreactivity in these samples and nuclear AR staining is proposed to reflect AR activity [158]. Evaluation of HLA class I ABC immunoreactivity in tumor cells showed an inverse correlation to nuclear AR score. Metastases with moderate to intense HLA class I ABC immunostaining also showed a significantly higher frequency of CD3$^+$ infiltrating cells compared to cases with negative or weak immunostaining. Due to observation of staining heterogeneity for both AR and HLA class I ABC we also performed double stainings which showed an inverse staining pattern between nuclear AR and HLA class I ABC. Immunostaining of AR co-regulator FOXA1 in consecutive sections showed reduced levels in AR-negative/HLA class I ABC–positive tumor cells.
In order to evaluate HLA class I expression as a prognostic marker in primary prostate cancer, HLA class I ABC immunoreactivity was also evaluated in a TMA including prostate cancer tissue obtained from 284 patients undergoing TURP with long clinical follow up. Adjacent benign tissue was available in 179 cases. Results showed less intense staining in malignant epithelial cells compared to adjacent benign epithelial cells and lower HLA class I ABC staining in tumor cells was associated with higher Gleason score and metastases at diagnosis (M1). Also when M1 patients were excluded, survival analysis showed that patients with negative or weak HLA class I ABC immunostaining had shorter cancer-specific survival compared to patients with moderate/intense immunostaining. A similar but nonsignificant trend was seen in patients managed with watchful waiting.

These results confirm previous studies showing that PSMB9, TAP1, and HLA class I molecules is expressed at lower levels in prostate cancer compared to benign prostate tissue [159-161]. We also present a novel finding that prostate cancer metastases show lower HLA class I expression than primary prostate tumors and that low tumor HLA class I ABC immunoreactivity at diagnosis is associated with a poor prognosis.

Our findings might thus be valuable to consider when choosing immunotherapy for individual prostate cancer patients. Although the intact MHC class I antigen presentation in non-AR-driven CRPC bone metastases might suggest that they will be susceptible for immunotherapy, the fact that vaccine-based therapies such as Sipleucel-T and PROSTVAC targets proteins transcribed from AR-stimulated genes, ACPP (PAP) and KLK3 (PSA), indicates that these therapies might not work due to the inverse correlation seen between MHC class I antigen presentation and AR-regulated genes. AR-driven CRPC metastases will probably also show poor response to these antigen-directed vaccines because even if they express PAP and/or PSA their tumor cells may have downregulated MHC class I antigen presentation. On the other hand, because non-AR-driven metastases express high MHC class I antigen presentation, immune cell infiltration and also CTLA4, PDC1 (PD-1) and S100A9, patients within this subgroup might actually be susceptible for immune checkpoint inhibitors (ipilimumab, pembrolizumab) and Tasquinimod. However, this study includes a limited amount of samples and the non-AR-driven subgroup is particularly small so the results presented here needs to be verified in larger cohorts, and preferably in prostate cancer patients enrolled in immunotherapy trials. Low serum PSA, MHC class I expression in tumor cells and the immune cell profile in metastasis tissue and blood could possibly be used as therapy-predicting markers. Because of possible heterogeneity between metastases within the same patients [162] it would be preferable if several metastases could be studied in patients with multiple metastases.
The molecular drivers behind the presented subgroups of CRPC bone metastases are not known and should be further explored. AR co-regulators FOXA1 and HOXB13 has previously been linked to AR cistrome reprogramming suggesting that they possibly contribute in development of AR-driven bone metastases [163]. The prostate-derived Ets factor SPDEF has a more controversial role in prostate cancer and has been reported both as tumor promoter as well as tumor suppressor in different systems and conditions [164, 165]. Reduced immune cytotoxicity through decreased presence of lymphatic vessels have been strongly associated with metastatic process in colorectal cancer [166]. We observed low levels of lymphatic endothelial cell marker LYVE1 in AR-driven metastases indicating a low lymphatic vessel density possibly contributing to the low immune-cell infiltration seen. Also, low levels of pro-inflammatory cytokines and chemokines could most likely contribute. T-cell activity can possibly be inhibited in non-AR-driven metastases via Tregs and MDSCs activated by anti-inflammatory factors. Downregulation of MHC class I antigen presentation molecules in prostate cancer might be due to structural defects or by epigenetic, transcriptional, or post-transcriptional regulation [167]. If so, treatment with IFN-γ and inhibitors of methylation or histone deacetylation could partly restore MHC class I expression [161, 168, 169]. The inverse correlation between AR activity and MHC class I expression is in line with previously reported suppressive effects of androgens on immune responses and studies showing increased intra-prostatic lymphocyte infiltration in prostate cancer patients after ADT [170-172]. All in all, our results support suggested strategies of combining ADT with immunotherapy [173].

In conclusion, most CRPC bone metastases show high AR activity, high metabolic activity and low cellular immune responses. A subgroup shows low AR and metabolic activity but high cellular immune response. Targeted therapies for these subgroups should be explored.
**Paper II**

**Bone cell activity in clinical prostate cancer bone metastasis and its inverse relation to tumor cell androgen receptor activity**

Novel therapies for CRPC and metastatic prostate cancer includes bone-targeting therapies, but therapy-predicting markers are lacking. Prostate cancer bone metastases are generally classified as osteoblastic (or sclerotic), showing increased bone volume/density compared to healthy bone although a mixed osteoblastic/osteolytic bone response may exist as osteolytic activity is also seen in metastatic prostate cancer patients [174-177]. The osteoblastic phenotype of bone metastases in prostate cancer patients indicates the possibility of an association between excessive bone formation and AR activities. Androgens stimulate AR signaling in osteoblasts, causing increased bone formation and ADT have been shown to reduce bone mass in prostate cancer patients. In addition, the osteoblastic phenotype of prostate cancer have been proposed to originate from intra-tumoral steroidogenesis in CRPC and thus preserved androgen levels and AR activity [109, 178, 179].

In this study, we aimed to investigate bone cell activity in relation to tumor cell AR activity in bone metastases obtained from prostate cancer patients (11 treatment-naïve and 28 castration-resistant, selected based on bone content) in order to gain novel insight into biological heterogeneities of possible importance for patient stratification into bone-targeting therapies.

In order to characterize ongoing bone cell activity in prostate cancer bone metastasis, we analyzed expression levels of a selected set of marker genes representing osteoclasts \( \text{ACP5}, \text{CTSK}, \text{MMP9} \), osteoblasts \( \text{ALPL}, \text{BGLAP}, \text{RUNX2} \) and osteocytes \( \text{SOST} \) in gene expression data from paper I and another previous study within our group [101, 180]. To capture the variation in bone cell activity among bone metastasis samples, a PCA model was built based on the transcript levels of these marker genes. The significant principal component explaining 64% of the variation was not caused by the bone tissue fraction in the samples, but was assumed to describe bone cell activity based on our chosen set of marker genes and from here on this score vector was used to describe ongoing bone cell activity in metastasis samples on a continuous scale.

While we observed varied expression of marker genes between samples, we saw a positive correlation of bone cell activity markers within samples indicating parallel activation of osteoblasts and osteoclasts in prostate cancer bone metastases. Immunohistochemical analysis of the osteoblast marker protein RUNX2 and the osteoclast marker protein TRAP (encoded by \text{ACP5} \) in FFPE tissue samples \( \text{n}=35 \) supported the PCA model by showing a positive correlation.
between RUNX2-positive cells and TRAP-positive cells lining bone surfaces in close contact to tumor cells. Also, a positive correlation was seen between RUNX2 and TRAP protein levels to corresponding gene expression levels and similar to the gene expression data, protein levels varied between individual samples while correlated within samples. We observed no clear difference in bone cell activity between treatment-naïve and castration-resistant bone metastases.

Histological examination of van Gieson-stained metastatic tissue sections showed a difference in ongoing bone cell activity between samples. But heterogeneous bone cell activity was also seen within samples and areas with newly formed, osteocyte-rich woven bone could be found in the same sample as areas of old, lamellar bone. Newly formed bone was commonly observed in bone marrow cavities rich in tumor cells, and cells positive for RUNX2 and TRAP was primarily found lining the surface of this newly found bone.

To investigate clinical characteristics possibly differing between patients with high or low bone cell activity we compared RUNX2 and TRAP immunoreactivity to a number of clinical variables. Both RUNX2 and TRAP was found to be inversely correlated to patient serum PSA at metastasis surgery, indicating that patients with high bone cell activity also have low serum PSA levels. Patient age at diagnosis was also correlated to RUNX2 and TRAP immunoreactivity, suggesting that older prostate cancer patients are more likely to have metastases showing high bone cell activity. Patients who had undergone chemotherapy before metastasis surgery (n=4) showed considerably lower RUNX2 immunoreactivity, but TRAP immunoreactivity showed no difference compared to patients previously untreated with chemotherapy. Although based on very few patients this finding might suggest that chemotherapy promotes an osteolytic response in bone. We found no other clinical variables significantly related between RUNX2 or TRAP immunoreactivity although a tendency of lower RUNX2 immunoreactivity was observed in patients who had received radiation towards the metastatic site and in patients previously treated with Ra-223. This possible promotion of osteolytic activity by chemotherapy, and maybe other treatments, should be further explored in a larger cohort of patients.

Furthermore, OPLS modeling was used to analyze the whole-genome expression profiles of metastases in relation to the bone cell activity score vector established in the earlier PCA analysis. In line with the inverse correlation seen between bone cell activity and serum PSA, we observed an inverse relation between bone cell activity and AR activity based on expression of the AR, AR co-regulators FOXA1 and HOXB13, and AR regulated genes KLK2, KLK3, NKX3-1, STEAP2, and TMPRSS2 (as defined in paper I [180]). The most prominent inverse correlation between bone cell activity and AR associated genes was seen for RUNX2 and this finding was also supported by a significant inverse correlation between RUNX2
immunoreactivity and \textit{KLK2}, \textit{KLK3} and \textit{HOXB13} gene expression. We observed no significant correlation between TRAP immunoreactivity and any of the examined AR associated genes. Taken together, these results indicates that bone cell activity is inversely correlated to tumor cell AR activity and patient serum PSA levels.

In order to search for possible drivers behind bone cell activity in prostate cancer metastases, genes with strong correlations to the bone cell activity score vector were imported into the MetaCore software for functional enrichment analysis. As expected, “ossification and bone remodeling” was found to be the most highly enriched process network. Other enriched networks involved includes epithelial-to-mesenchymal transition (EMT), cell adhesion, proliferation, bone morphogenetic protein (BMP) and growth differentiating factor (GDF) signaling, cartilage development and inflammation. We found no significantly enriched process to be negatively associated to the bone cell activity score vector.

To identify possible osteoclast/osteoblast regulators originating from tumor cells, probable upstream regulators identified by the MetaCore software (based on known protein interactions from the literature) were analyzed in relation to ongoing bone formation in metastasis samples by OPLS discriminant analysis (OPLS-DA) modeling. Class membership was set as ongoing or no ongoing bone formation, based on histological examination of van Gieson-stained tissue sections. Patients previously treated with chemotherapy, radiotherapy or radiation towards operation site were excluded from this analysis, only treatment-naïve or primarily castration-resistant patients were considered.

Although the resulting OPLS-DA model did not fully separate metastases with active bone formation from those without active bone formation, it did show a significant correlation to osteocyte content and could therefore be used to find gene products capturing variations between classes. \textit{BMP2} and \textit{BMP4} was found among the suggested regulators highly expressed in cases with active bone formation indicating an increased BMP signaling in these patients. Tumor cells can secrete BMPs and other osteogenic growth factors in order to increase metastasis growth and aggressiveness via a so called vicious cycle [26, 181].

Osteoclast differentiation and bone formation are known to be stimulated by \textit{BMP2} and \textit{BMP4} via bone specific transcription factors such as \textit{RUNX2}, \textit{DLX5} and \textit{SP7} [182]. Besides bone formation, BMPs might also be involved in some other processes, such as cell proliferation, differentiation, apoptosis and angiogenesis [183, 184]. \textit{BMP4} has been show to promote prostate tumor growth by stimulation of osteogenesis and also to induce conversion of tumor-associated endothelial cells into osteoblasts [185, 186] \textit{BMP2} has been suggested to be involved in induction of EMT and inhibition of tumor cell apoptosis [187]. \textit{BMP2} signaling is also known to activate \textit{SPP1} via the RUNX2-SMAD complex [188].
The *SPP1* gene encodes for osteopontin, a major non-collagenous protein in bone matrix promoting bone resorption by facilitating the binding of osteoclasts to bone [189, 190]. Osteopontin overexpression has been shown to increase proliferation and invasiveness in LNCaP prostate cancer cells [191]. Osteopontin has also been shown to instigate the growth of indolent metastases in a breast cancer model [192].

Results from the OPLS-DA showed an indication of ongoing BMP (and/or possibly TGF-β) signaling based on high expression of *DCN, DLX3, DLX5, BGN, ZEB2, FST, and SMAD7* in metastases with high expression of *RUNX2, SPP1* and other genes associated with osteoblastogenesis and bone formation such as *BGLAP, SATB2, SPHK1, COL1A1, COL1A2, PHEX, SP7, ALPL, IBSP*, and *SOST* as well as detectable bone formation [193-201].

Metastatic tumor cells was identified as the predominant source of *BMP4* by immunohistochemistry showing specific BMP4 immunoreactivity in tumor cells and while positive BMP4 immunoreactivity also was seen in endothelial cells, lipocytes and cells lining bone surface these findings were not further assessed due to limited variability between patients. Moreover, most of the metastases with active bone formation (89%) showed higher BMP4 immunoreactivity, also in line with *BMP4* transcript levels, compared to metastases without active bone formation. The cell origin of *BMP2* was not further examined.

Inverse correlations were found between the *BMP4* and *BMP2* transcript levels and several AR associated gene transcripts such as *KLK3, STEAP2, FOXA1, HOXB13* and *NKX3-1*. Also, *BMP4* and *BMP2* expression showed a significantly inversed correlation to patient serum PSA at metastasis surgery. Taken together, these results indicate a positive correlation between BMP signaling, bone cell activity and pathologic bone formation in prostate cancer bone metastases and, in contrast to what could have been expected, those processes are suggested to be negatively correlated to tumor cell AR activity and patient serum PSA.

Other pathways previously suggested to be involved in bone remodeling is the WNT signaling system and the osteolytic factor PTHRP [202, 203]. However, we found low expression levels of factors involved in WNT signaling as well as of *PTHRP* in all metastatic samples included in this study indicating no obvious role for these pathways in stimulating bone cell activity in prostate cancer bone metastases.

Bone resorption can be targeted using bisphosphonates, which inhibits osteoclasts and osteolysis and has been proven to reduce the incidence of SREs in patients with prostate cancer bone metastases [204]. Another treatment targeting bone resorption is Denosumab, a monoclonal antibody inhibiting the RANKL protein which is a mediator of osteoclaogenesis and bone resorption [205]. However, *TNFSF11* (encoding for RANKL) and its neutralizing decoy
receptor TNFRSF11B (OPG) could not be detected in most samples included in this study and immunohistochemistry would probably be needed to assess whether these factors are present in metastasis tissue and their relation to osteoclast activity. Another bone-targeting therapy possibly suitable for prostate cancer patients whose bone metastases show high bone cell activity is radium-223, a α particle-emitting agent acting as a calcium mimetic, therefore accumulating in areas with high bone turnover where it is incorporated into bone [70, 71].

Based on their functions in bone remodeling and cancer, BMPs could also be considered as possible therapeutic targets. Noggin is a natural BMP inhibitor found to inhibit both BMP2 and BMP4, and also to inhibit expansion of PC-3 prostate cancer cells in vivo [206]. Also, ovarian cancer cell growth has been showed to be inhibited in vivo by the small molecule inhibitor of type I BMP receptors Dorsomorphin [207].

Limitations of this study include the relatively low number of samples analyzed and the examination of only one metastasis sample per patient. Also, the net effect of high bone cell activity on bone formation could not be clarified. Evaluation of bone cell activity in relation to matched radiography data and serum samples for circulating biomarker analysis would have been helpful, but such data were not available. Therefore, the possible value of the molecular findings in this study should be further explored in clinical studies where bone metastasis samples (optimally sampled from several sites) are collected in parallel with radiography, MR and bone scan data and serum samples prior to treatment with bone-targeting therapies. In such studies, bone cell activity in metastasis tissue could be evaluated in relation to circulating markers for bone remodeling (reviewed in [208]) as well as the osteolytic/osteoblastic metastasis phenotype and also to patient response to therapy. A possible correlation between circulating markers for bone remodeling, bone cell activity in metastasis tissue, and therapy response would mean that these circulating markers could be used as therapy-predicting biomarkers.

As stated earlier, intra-tumoral steroidogenesis have been proposed to contribute to the osteoblastic phenotype seen in metastatic prostate cancer. Notably, results from our previous study examining expression of steroidogenic enzymes in the same patient material showed that bone metastases with ongoing bone formation showed higher tumor cell AKR1C3 immunoreactivity than cases without detectable bone formation [113], possibly due to intra-tumoral conversion of androstenedione to testosterone and subsequent osteoblast activation. Therefore it would also be interesting to measure intra-metastatic levels of androgens to further explore if the excessive bone formation might be related to restored androgen levels due to intra-tumoral steroidogenesis. Also, because activation of the estrogen receptor α (ERα) have a similar effect on bone formation as the AR it could perhaps be of interest to measure intra-metastatic estrogens [179].
In conclusion, this study shows that bone cell activity varies between patients with metastatic prostate cancer. Specifically, high bone cell activity was observed in non-AR driven bone metastases in patients with low serum PSA levels, while low bone cell activity was seen in AR-driven metastases in patients with high serum PSA levels. These findings might be of importance considering stratification of metastatic prostate cancer patients into AR- and/or bone-targeting therapies.

Furthermore, this study adds information to the two subgroups among CRPC bone metastases identified in paper I; those that have high AR and metabolic activity, but show low cellular immune responses, and those with low AR and metabolic activity, but more prominent immune responses, by finding high bone cell activity primarily in patients with low AR activity and ongoing inflammation.
Integrated DNA methylation and gene expression analysis of molecular heterogeneity in prostate cancer bone metastasis

In order to guide further diagnostic and therapeutic developments, a better understanding of the tumor biology in prostate cancer bone metastases is needed. In previous studies, we have explored the transcriptome and proteome in prostate cancer patient bone metastases and identified molecular heterogeneities that differentiate bone metastasis samples into metastasis subtypes associated with tumor biology and patient prognosis [77, 101, 113, 143, 180, 209] (and recently submitted work by our group, Thysell et al. 2018). Specifically, paper I and II of this thesis describe heterogeneities related to tumor cell AR activity [180, 209]. The mechanisms underlying the development of metastasis subtypes are currently unknown, but may be related to epigenetic dysregulation affecting differentiation and clonal expansion. Although the knowledge about DNA methylation alterations in prostate cancer is limited, gene hypermethylation has been described to contribute to prostate cancer progression [131].

In this paper, we have explored DNA methylation patterns of prostate cancer bone metastases (n=70, of which 14 previously untreated hormone-naive, 4 short-term castrated and 52 CRPC) in relation to molecular heterogeneity observed at the RNA expression level. Specifically we have aimed to study promotor gene methylation levels in relation to tumor cell AR activity defined from expression levels of genes associated with canonical AR activity (as defined in paper I, [180]). In addition, we have compared the general methylation pattern of bone metastases to patterns seen in paired samples of non-malignant prostate and primary prostate cancer from a separate patient cohort (n=13).

In order to investigate gene methylation status we have used genome-wide methylation arrays and to facilitate correlation studies between methylation levels of gene promoters and corresponding transcript levels, we have focused the analysis on methylated CpG-sites (mCpGs) within promoter regions.

PCA modeling based on these promotor-associated mCpGs showed a clear separation between non-malignant, primary prostate cancer and metastasis samples, with the exception of few samples, one non-malignant, two primary tumors and two metastasis, of which the non-malignant sample turned out to be misclassified, by showing infiltration of malignant cells at histological re-examination. We could not find any obvious explanation by histologically examining the rest of the samples which clustered seemingly incorrect. Further morphological analysis showed a lower fraction of epithelial cells in non-malignant samples compared to primary tumors and metastases. Also, no obvious relation of metastasis clustering to epithelial cell fraction or to previous treatment was seen.

Differentially methylated CpG sites (DM-CpGs) between non-malignant and primary tumor samples were analyzed in comparison to mRNA transcript profiles.
from paper I [180] and other previous work from our group [101]. Genes in primary tumors showing a hypermethylation, with a corresponding decrease in gene expression, and hypomethylated genes showing increased expression levels were extracted for further analyzation by OPLS-DA and GSEA. The results showed a pronounced hypermethylation in primary prostate cancer associated with reduced expression of genes involved in immune responses and developmental processes while hypomethylated genes did not show significant association to any available functional pathway within the analysis software.

These results are in line with previous studies, summarized in [131], suggesting hypermethylation as an early event in prostate cancer tumorigenesis and also proposing that it could possibly be used for diagnostic purpose. Among hypermethylated genes, we found GSTP1 and other genes known to be hypermethylated in prostate cancer including AOX1, BARHL2, CCDC8, CDKN2A, CYP27A1, EFS, GRASP, HOXA3, HOXC11, HOXD3, KIT, NXX2-1, NXX2-5, PHOX2A, POU3F3, PTGS2, RARB, RHCG, SIX6, TBX15, TMEM106A, WNT2, and ZNF154 [131] of which PTGS2 and HOXD3 have been suggested to provide prognostic information [210, 211]. However, due to the low number of primary tumor samples we could not evaluate DM-CpGs in relation to prognosis. Instead, we focused further analysis on identifying general effects of methylation changes during prostate cancer progression.

The results from the GSEA indicated that reduced STAT3 and STAT5 expression in primary tumors, presumably caused by the observed hypermethylation, may contribute to the suppressive immune phenotype repeatedly observed in prostate cancer, including low tumor infiltration of CD4/CD8\(^+\) effector T cells and high infiltration of suppressive immune cells such as FoxP3\(^+\) regulatory T cells, CD163\(^+\) macrophages, and S100A9\(^+\) inflammatory cells [150-154, 212]. However, the reduced immune cell response and other pathways (endothelial cell differentiation and TGF-\(\beta\) induced EMT) suggested by GSEA to be down-modulated in primary tumors due to methylated gene promoters could also be biased due to the non-malignant samples having a higher stroma content than the samples from primary tumors. Although hypomethylation in primary tumors was shown to be uncommon and no association with any functional pathway was found by the analysis software, we did notice some hypomethylated genes frequently reported in prostate cancer, such as ESR1 coding for the estrogen receptor alpha [131].

To further analyze DM-CpGs possibly related to disease progression, we focused on DM-CpGs between primary tumors and metastases while DM-CpGs between non-malignant and metastasis samples were not further explored because they were likely to be organ related. Most DM-CpGs observed between primary prostate cancer and non-malignant prostate tissue appeared to be maintained in the metastatic tissue but to explore this further paired primary tumor and metastases samples need to be examined. However, when analyzing hypomethylated genes with consistently increased expression levels in
metastases, we found that many AR promotor associated CpGs showed decreased methylation levels in metastases compared to primary tumors as well as non-malignant prostate, and the two most frequently hypomethylated CpGs were located close to two alternative AR transcription start sites. Accordingly, AR transcript levels were also higher in metastases samples compared to primary tumors and non-malignant prostate.

High AR expression in CRPC have been previously associated with AR amplification [73, 75, 77, 213, 214], while methylation as a regulator of AR expression is less well described and previous studies have shown inconsistent results [134, 215, 216].

The findings in this study indicating demethylation of the AR promotor in CRPC as well as in hormone-naïve prostate cancer metastases suggests that AR demethylation could be an earlier cause to AR induction in prostate cancer metastases than AR amplification, as AR amplification is generally not seen before ADT. Moreover, the large variance in AR methylation levels observed among the metastasis samples deserves to be further explored as it might be related to tumor cell AR dependence.

To study the relation of promotor methylation to AR activity, we gave each individual metastasis sample an AR activity score based on expression levels of AR regulated genes, as defined in paper I, [180]. All mCpGs positively or negatively correlated to this score were subjected to further exploration and cluster analysis of these DM-CpGs separated metastasis samples into two main clusters. Taken together, metastases within the larger cluster 2 (i.e. AR-driven cases) showed low promotor methylation levels of several AR-regulated genes (KLK2, SLC45A3, STEAP2, and TRMPSS2) and AR co-factors (NCOA2 and NCOA3) possibly contributing to the AR-driven tumor phenotype seen in most of metastatic prostate cancer patients, described in paper I [180] and other recently submitted work from our group (Thysell et al. 2018). Accordingly, patients in cluster 2 showed higher PSA levels in both serum and tumor tissue and also a longer survival after ADT, probably because of their AR-driven tumor cells responding better to ADT than patients in the smaller cluster 1 (i.e non-AR-driven metastases).

Because metastases within cluster 1 were specified by hypermethylation of AR-regulated genes and co-factors, it is tempting to speculate that treatment using demethylating agents could be a possible strategy to sensitize them to AR-targeting therapies, as have been shown possible in different experimental systems for CRPC [134, 135, 217, 218]. A recent study identified the histone lysine-N-methyltransferase EZH2 as an important epigenetic regulator of AR-targeting therapy resistance and also suggested EZH2 inhibition as a potential CRPC treatment [219]. Also, there is a hypothetical possibility to predict patient response to AR-targeting therapies by evaluating the methylation pattern of a selected set of mCpGs.
To specifically examine if the low immune cell responses in AR-driven metastases previously observed in paper I [180] could possibly be related to altered promotor methylation of genes involved in antigen presentation, we extracted data for MHC class I and II, PSMB8, and TAP1/2 genes from metastasis samples.

The strongest correlations between AR activity scores and methylation levels were found for CpGs in the promotor region of PSMB8, HLA-C, HLA-DPA1, and HLA-DMB genes. Although samples in cluster 2 showed parallel reductions in transcript levels for these genes as well as lower CD3+ T cell and CD68+ macrophage infiltration, further inspection of all CpGs in PSMB8, HLA-DMB, and HLA-DPA1 promoters showed only modest differences between metastasis samples of cluster 1 (non-AR-driven) and 2 (AR-driven) suggesting that other mechanistic explanations behind down-modulated antigen presentation in prostate cancer bone metastases probably exist.

In conclusion, this study describe general patterns of gene promotor methylations during prostate cancer progression and possible functional effects on AR signaling and immune cell responses. Furthermore, based on methylation levels in promotor regions of genes associated with AR signaling, metastatic prostate cancer patients could be divided into two clusters with different prognosis. The biological relevance and possible clinical usefulness of the mCpG changes observed during prostate cancer progression in this study deserves further exploration. The overall cause to dysregulated gene methylation during prostate cancer progression also remains to be identified.
Paper IV

Excellent cabazitaxel response in prostate cancer xenografts expressing androgen receptor variant 7 and reversion of resistance development by anti-androgens

New treatments for CRPC are constantly under development and in recent years several novel therapies, have been approved including abiraterone, a steroidogenesis inhibitor, and cabazitaxel, a second-generation taxane. However, because several different mechanisms have been suggested to be involved in CRPC development and the newly developed therapies are based on different mechanisms of actions, therapy response will probably be determined by the molecular properties of individual CRPC patient tumors. Also, any effective therapy will probably induce molecular adaptations leading to subsequent therapy resistance and tumor relapse.

Among the proposed mechanisms underlying CRPC, the expression of constitutively active AR variants have been of particular interest in recent years. In a previous study, we showed that a subgroup of CRPC bone metastases in patients expresses high levels of constitutively active AR variants; in particular AR-V7, which was associated with a very poor prognosis [101].

In this study, the aim was to investigate the effects of abiraterone and cabazitaxel, and subsequently developed resistance mechanisms, in human 22Rv1 prostate cancer xenografts, expressing high levels of constitutively active AR variants, including AR-v7.

We established xenografts by subcutaneously inoculating nude mice with 22Rv1 cancer cells and when tumors had reached an appropriate size mice were treated with surgical castration, abiraterone (+/− castration) or cabazitaxel (+/− castration). Mice in the control group received vehicle and/or sham operation. The results showed no significant effect of abiraterone while castration with or without abiraterone showed a modest reduction in tumor growth rate. Cabazitaxel, on the other hand, retarded tumor growth both with and without castration. However, tumor regrowth was seen after some time in the majority of cabazitaxel treated animals and a subset of mice treated with cabazitaxel (+castration) were repeatedly treated until resistance occurred.

These results are largely in line with previous studies showing that CRPC patients with detectable levels of AR-V7 in CTCs or blood are likely to be resistant towards abiraterone and other AR-targeting therapies while taxanes, such as cabazitaxel, are more suitable treatment options for these patients [103-108]. Surgical castration effects that moderately reduced growth of 22Rv1 xenografts could probably be explained by reduced testosterone levels and inhibition of the full-
length AR activation. Why abiraterone is less effective, despite showing a castrate effect manifested as reduced ventral prostate weight, is not known.

To study the mechanisms behind cabazitaxel resistance, we established cell lines from two of the 22Rv1 xenografts showing resistance towards cabazitaxel. The resulting cell lines, 22Rv1-CabR1 and 22Rv1-CabR2, were subjected to further selection *in vitro* and a dose-response experiment confirmed their cabazitaxel resistance by showing a 30-fold higher IC$_{50}$ value for 22Rv1-CabR1 and 22Rv1-CabR2 compared to 22Rv1. *In vivo* cabazitaxel resistance was tested by an additional round of nude mice xenografts, this time injected with 22Rv1-CabR1, 22Rv1-CabR2, or 22Rv1, and all mice were given the same treatment; cabazitaxel combined with castration. Resistance was confirmed by 22Rv1-CabR1 and 22Rv1-CabR2 xenografts growing rapidly while a clear regression was seen for the control 22Rv1 xenografts.

In order to identify common trends in expression patterns for 22Rv1-CabR1 and 22Rv1-CabR2 cell lines in comparison to cabazitaxel responsive 22Rv1 cells, we used multivariate PCA modeling to analyze gene expression data. The resulting PCA plot showed that both 22Rv1-CabR1 and 22Rv1-CabR2 have highly induced transcript levels of *ABCB1* compared to 22Rv1. The *ABCB1* gene codes for the ATP-binding cassette sub-family B member 1 (ABCB1) protein, also known as multidrug resistance protein 1 (Mdr1) or P-glycoprotein (P-gp), a membrane bound transporter protein known to function as a drug efflux pump contributing to resistance towards several drug, including taxanes [220]. Overexpression of the ABCB1 protein was confirmed *in vitro* by western blot showing upregulation of ABCB1 in 22Rv1-CabR1 and 22Rv1-CabR2 cells and *in vivo* by immunohistochemical analysis showing an increased ABCB1 membrane staining in 22Rv1-CabR1 and 22Rv1-CabR2 xenografts compared to 22Rv1 controls. Treatment with the ABCB1 inhibitor elacridar restored the cabazitaxel susceptibility in 22Rv1-CabR1 and 22Rv1-CabR2 cells to similar levels as 22Rv1 control cells, thereby confirming that cabazitaxel resistance in these cells is caused by ABCB1 overexpression.

Several ABCB1 inhibitors, including elacridar, have been developed over the years to counteract ABCB1 mediated taxane resistance, but so far none have made it through clinical trials [221]. In spite of the fact that cabazitaxel was specifically chosen for clinical development based on its activity taxane resistant pre-clinical models, including cell lines overexpressing ABCB1 [64], a recent study has showed that cross-resistance between docetaxel and cabazitaxel can be mediated through ABCB1 overexpression in docetaxel resistant prostate cancer cell lines [222]. Remarkably, that study, and earlier work from the same group, also showed that inhibition of ABCB1 efflux activity, and thus restored taxane sensitivity, can be achieved by treating cells with anti-androgens; bicalutamide.
and enzalutamide [222, 223]. For that reason, we also treated the cabazitaxel resistant cell lines in this study, 22Rv1-CabR1 and 22Rv1-CabR2, with these anti-androgens and the results showed that both bicalutamide and enzalutamide significantly increased the susceptibility to cabazitaxel although none of them were quite as effective as elacridar. Neither anti-androgens nor elacridar did show any additional effect to cabazitaxel in the 22Rv1 control cells.

Surprisingly, 22Rv1-CabR1 and 22Rv1-CabR2 also showed inductions of AR-regulated genes such as NKX3-1 (also confirmed by western blot) and STEAP1, while other gene expression patterns were more diverse. Western blot analysis indicated a decreased expression of AR and an increase of AR-Vs in cell lines cultured in charcoal-stripped media compared to 22Rv1 grown in regular media, but AR-V expression did not obviously change due to cabazitaxel resistance. Similar results was seen in vivo; castrated 22Rv1 xenografts showed reduced AR protein expression compared to non-castrated, while AR-V protein showed little or no change due to other treatments. A larger decrease of AR protein expression, but not AR-V, was seen in 22Rv1-CabR1 compared to 22Rv1-CabR2 and 22Rv1 cells, which was confirmed by a similar decrease in nuclear AR immunostaining in 22Rv1-CabR1 xenografts, while no difference was seen for AR-V7 immunostaining in relation to cabazitaxel resistance.

That AR-V expression did not change between treatments in 22Rv1 xenografts or in cabazitaxel resistant cell lines or xenografts indicates that AR variants, including AR-V7, are not involved in the development of taxane resistance. This finding is in line with a recent study where overexpression of AR-V7 in C4-2B prostate cancer cells did not induce resistance to cabazitaxel or docetaxel [224]. Notably, while ABCB1 inhibition by anti-androgens is thought to be independent of AR-status [223], bicalutamide and enzalutamide were both more effective in restoring susceptibility to cabazitaxel in 22Rv1-CabR2, with intact expression of full-length AR, than 22Rv1-CabR2 cells, in which reduced expression of the full-length AR was observed.

Taken together, our results confirm previous studies suggesting a combination of taxanes and antiandrogens to overcome ABCB1-mediated taxane resistance in CRPC.

Pathway enrichment analysis of differently expressed gene transcripts with a fold-change ≥2 (P<0.05) showed an increase of the SCAP/SREBP transcriptional control of cholesterol and fatty acid biosynthesis in 22Rv1-CabR2 cells, but not 22Rv1-CabR1, compared to 22Rv1. This is a finding in line with previous studies from our group showing high cholesterol levels and β-oxidation in CRPC metastases [142, 143, 180]. In light of this finding, we treated the cells with simvastatin, a cholesterol lowering drug with potential beneficial effects on
prostate cancer patients [225]. Dose-response experiments indicated a reduced sensitivity to simvastatin in 22Rv1-CabR2 by showing an IC<sub>50</sub> value 3-5 times higher than 22Rv1-CabR1 and 22Rv1, presumably caused by up-regulation of molecules within the targeted pathway. Nevertheless, treatment using simvastatin and other drugs targeting this pathway might be a suitable option in CRPC cases with features similar to 22Rv1.

PCA analysis of xenograft tumors relapsing after cabazitaxel treatment showed diverse gene expression patterns and ABCB1 upregulation was seen in two xenografts only, the two that were subsequently established as cell lines. The rest of the cabazitaxel resistant xenografts did not show any evident increase in ABCB1 or any other efflux transporters, indicating other underlying mechanisms in these cases. Other groups have reported taxane resistance mechanisms including accumulation of β-tubulins, alterations of survival factors and regulators of apoptosis, downregulation of BRCA1, and EMT [223, 226]. We found no significant evidence suggesting these mechanisms being active in cabazitaxel resistant 22Rv1 xenografts, although scattered and modest changes were observed for some genes involved in the proposed processes, such as TUBB2A, TUBB2B, TUBB3, BRCA1 and VIM1. However, no cell lines were established from these xenografts, so we did not further examine other cabazitaxel resistance mechanisms than induced ABCB1 expression and induced cholesterol biosynthesis. While a strong consensus was seen for decreased expression of cell cycle regulating genes in cabazitaxel resistant xenografts we assume it to be related to ongoing chemotherapy.

The toxicity of taxanes in general and cabazitaxel specifically restrict the use in patients with co-morbidity. The results that bicalutamide, an inexpensive and well tolerated drug, may sensitize the tumor cells for cabazitaxel, raise the question if adjuvant treatment with bicalutamide to reduce the cabazitaxel dose could be a therapeutic option for these patients. Even a modest reduction of cabazitaxel dosage may be clinically very relevant in patients with co-morbidity who otherwise would be denied life-prolonging chemotherapy.

In conclusion, this study shows great initial response to cabazitaxel in the 22Rv1 xenograft model of CRPC expressing constitutively active, LBD-truncated AR variants (including AR-V7). The later developed resistance to cabazitaxel was in some cases related to overexpression of the ABCB1 drug efflux transporter as well as by increased cholesterol biosynthesis. Besides more specifically developed ABCB1 inhibitors such as elacridar, ABCB1-mediated cabazitaxel resistance could be partly overcome by co-administration of AR-antagonists bicalutamide and enzalutamide.
Conclusions

Paper I & II

- The majority of prostate cancer bone metastases are apparently AR-driven, based on their high expression of AR regulated genes.
- AR-driven metastases are characterized by:
  - High metabolic activity
  - High patient serum PSA
  - May benefit from AR-targeting therapies and/or drugs targeting metabolic pathways
- A smaller sub-group of bone metastases are apparently non-AR-driven, based on their low expression of AR regulated genes.
- Non-AR-driven metastases are characterized by:
  - Low patient serum PSA
  - High immune responses
  - High bone cell activity
  - May benefit from immunotherapy and/or bone-targeting therapies

Paper III

- Pronounced hypermethylation during prostate cancer progression, possibly related to a suppressed immune-phenotype
- Prostate cancer metastases could be divided into two clusters based on methylation levels in gene promoters associated with AR signaling:
  - Larger cluster: higher AR activity and better prognosis
  - Smaller cluster: lower AR activity and worse prognosis

Paper IV

- 22Rv1 xenografts show excellent response to cabazitaxel and poor response to abiraterone
- Cabazitaxel resistance eventually develops in responding cells and is associated with overexpression of the multidrug efflux pump ABCB1
- AR-antagonists bicalutamide and enzalutamide partly restores susceptibility to cabazitaxel
- Increased cholesterol and fatty acid synthesis in cabazitaxel resistant cells reduces susceptibility to simvastatin
Concluding remarks

In this thesis, we have identified molecular subgroups of prostate cancer bone metastases by analyzing whole-genome expression patterns. Based on different expression patterns of AR regulated genes, we show that a majority of prostate cancer bone metastases are apparently AR-driven while a smaller sub-group is considered non-AR-driven, supported by a corresponding difference in patient serum PSA. Additionally, molecular differences in metabolic activity, cellular immune response and bone cell activity also differ between these two subgroups.

In addition to gene expression data, we verified our findings at the protein level and also show that they are of prognostic value. In theory, increased knowledge on these subgroups could hopefully contribute to the development of treatment predictive markers and subtype-specific treatments, but this remains to be proven by further studies.

Furthermore, we have shown that although the novel chemotherapeutic drug cabazitaxel is initially efficient in treating prostate cancer expressing truncated AR variants, resistance eventually develops and anti-androgens could be a potential strategy in order to revoke cabazitaxel resistance.

The mechanisms underlying the different gene expression patterns could possibly be explained by epigenetic changes in some genes but can probably also be affected by genetic defects, a limitation of this thesis is that we have not explored our samples on the DNA level so we cannot answer that question. Another obvious weakness is that we have not included more than one metastasis sample per patient so we are not able to further explore metastasis heterogeneity within individual patients. The possibility of heterogeneity within individual metastases also need to be explored.

Taken together, this thesis shows that molecular heterogeneity in prostate cancer bone metastases may be of importance for future patient treatment stratification and development of novel therapeutics, hypothetically targeting the tumor phenotypes as indicated in Figure 5.
Figure 5: Concluding remarks. This thesis suggests two subgroups of prostate cancer bone metastases. AR-driven metastases (upper): high AR-activity, high metabolic activity and high patient serum PSA. Non-AR-driven metastases (lower): low AR-activity, low patient serum PSA, high cellular immune responses and high bone cell activity. Subgroups could possibly be explained by different changes in promotor methylation during prostate cancer progression. Based on the molecular properties of these subgroups, AR-driven metastases will probably benefit from ADT and AR-targeting therapies and possibly also metabolic drugs. Non-AR-driven metastases will less likely respond to ADT but maybe instead be more susceptible to immunotherapies and bone-targeting therapies. Chemotherapies could be an effective treatment option for both subgroups.
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