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Evidence from preclinical models and clinical specimens

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to my family

ABSTRACT

Matias Knuuttila

Intratumoral androgen biosynthesis in prostate cancer: Evidence from preclinical models and clinical specimens

University of Turku, Faculty of Medicine, Institute of Biomedicine, Physiology, Drug Research Doctoral Programme (DRDP)

Annales Universitatis Turkuensis, Medica-Odontologica, Turku, 2017

Androgens regulate the growth and development of normal prostate and prostate cancer. Blocking the production and effects of androgens, either by castration or medication, has been the most efficient strategy for treating metastatic prostate cancer for decades. Although most patients respond to the therapy, in many of them the disease progresses to castration-resistant prostate cancer (CRPC) that cannot be cured with current therapies. Intratumoral androgen biosynthesis has been identified as one of the mechanisms leading to castration resistance. Recent studies have confirmed that prostate tumors can synthesize androgens by themselves to maintain tumor growth in androgen-deprived conditions. Thus, suppressing intratumoral androgen biosynthesis has become an attractive option for drug development. To understand the mechanisms of intratumoral androgen biosynthesis and develop new CRPC therapies, better preclinical models for CRPC are needed.

In this study, we developed an orthotopic VCaP xenograft model suitable for studying the progression of CRPC and the mechanisms of intratumoral androgen biosynthesis *in vivo*. The VCaP model exhibited the clinical features of CRPC, including the activation of intratumoral androgen biosynthesis and the overexpression of androgen receptor (AR) and its splice variants. Furthermore, novel antiandrogens enzalutamide and ARN-509 reduced intratumoral androgen levels and altered steroidogenic enzyme expression in the VCaP model. In addition to preclinical studies, androgen levels were analyzed in prostate and serum specimens obtained from prostate cancer patients. Intraprostatic androgen levels in cancerous and benign samples were highly variable between the patients. Distinct intratumoral androgen levels and altered AR target gene expression were associated with TMPRSS2-ERG fusion gene expression.

Keywords: intratumoral, androgen, prostate cancer, xenograft

TIIVISTELMÄ

Matias Knuuttila

Kasvaintensisäinen androgeenituotanto eturauhassyövässä: Uutta tietoa eläinmalli- ja potilastutkimuksista

Turun Yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Fysiologia, Lääketutkimuksen tohtoriohjelma (DRDP)

Annales Universitatis Turkuensis, Medica-Odontologica, Turku, 2017

Androgeenit eli mieshormonit säätelevät eturauhasen ja eturauhassyövän kasvua. Androgeenien tuotannon ja vaikutusten estäminen joko kastraation tai lääkkeiden avulla on ollut tehokkain hoitomuoto levinneeseen eturauhassyöpään jo vuosikymmeniä. Vaikka suurimmalla osalla potilaista hoito tehoaa, merkittävällä osalla eturauhassyöpä kehittyy kastraatioresistentiksi eturauhassyöväksi, johon ei ole parantavaa hoitomuotoa. Kasvaintensisäistä androgeenituotantoa pidetään yhtenä mekanismina, joka mahdollistaa eturauhassyövän kehittymisen kastraatio-resistentiksi. Viimeaikaiset tutkimukset ovat osoittaneet, että eturauhassyöpä-kasvaimet pystyvät itse tuottamaan androgeeneja ylläpitääkseen eturauhassyövän kasvua, minkä vuoksi kasvaintensisäisen androgeenituotannon estämisestä on tullut varteenotettava vaihtoehto uusille lääkehoidoille. Jotta kasvaintensisäisen androgeenituotannon mekanismeja ymmärrettäisiin paremmin, tarvitaan parempia eläinmalleja uusien lääkkeiden vaikutuksien tutkimiseen.

Olemme kehittäneet ortotooppisen VCaP-ksenograftieläinmallin, jonka avulla mallinnamme eturauhassyövän kehittymistä kastraatioresistentiksi ja tutkimme kasvaintensisäistä androgeenituotantoa. VCaP-eläinmalli havainnollistaa monia kastraatioresistentille eturauhassyövälle tunnusomaisia piirteitä kuten kasvainten-sisäisen androgeenituotannon aktivoitumista ja androgeenireseptorin (AR) ja sen silmukointivarianttien yli-ilmentymistä. Uusien antiandrogeenien (entsalutamidi ja ARN-509) havaittiin laskevan kasvaintensisäisiä androgeenipitoisuuksia sekä muuttavan steroideja tuottavien entsyymien ilmentymistasoja VCaP-eläinmallissa. Eläinmallilla tehtyjen tutkimusten lisäksi mittasimme androgeeni-pitoisuuksia eturauhassyöpäpotilaiden eturauhaskudos- ja verinäytteistä. Androgeenipitoisuudet sekä eturauhassyöpänäytteissä että terveissä eturauhas-näytteissä vaihtelivat huomattavasti potilaiden välillä. Erilaiset kasvaintensisäiset androgeenipitoisuudet ja AR:n kohdegeenien ilmentymistasot osoittautuivat liittyvän TMPRSS2-ERG -fuusiogeenin ilmentymiseen.

Avainsanat: kasvaintensisäinen, androgeeni, eturauhassyöpä, eläinmalli

TABLE OF CONTENTS

ABBREVIATIONS

ADT	androgen deprivation therapy
AR	androgen receptor
ARE	androgen response element
BPH	benign prostate hyperplasia
CRPC	castration-resistant prostate cancer
DBD	DNA-binding domain
GC-MS/MS	gas chromatography-tandem mass spectrometry
ECL	electrochemiluminescence
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
GR	glucocorticoid receptor
HPG	hypothalamic-pituitary-gonadal
IB	immunoblotting
IHC	immunohistochemistry
LBD	ligand-binding domain
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LLOQ	lower limit of quantitation
LH	luteinizing hormone
mCRPC	metastatic castration-resistant prostate cancer
MR	mineralocorticoid receptor
NSAA	non-steroidal antiandrogen
ORX	orchidectomy
PSA	prostate-specific antigen
PDX	patient-derived xenograft
PIN	prostatic intraepithelial neoplasia
PR	progesterone receptor
RALP	robotic-assisted laparoscopic radical prostatectomy
RNA-seq	RNA sequencing
SD	standard deviation
SHBG	sex hormone-binding globulin
TNM	TNM classification of malignant tumors
TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
TRT	testosterone replacement therapy
UDPGA	uridine 5'-diphospho-glucuronic acid

LIST OF ORIGINAL PUBLICATIONS

The doctoral thesis is based on the following publications, which are referred to in the thesis using Roman numerals: I, II, III or IV.

I. **Knuuttila M**, Yatkin E, Kallio J, Savolainen S, Laajala TD, Aittokallio T, Oksala R, Häkkinen M, Keski-Rahkonen P, Auriola S, Poutanen M, Mäkelä S. Castration induces up-regulation of intratumoral androgen biosynthesis and androgen receptor expression in an orthotopic VCaP human prostate cancer xenograft model. *Am J Pathol.* (2014) 184:2163-2173.

II. Laajala TD, Jumppanen M, Huhtaniemi R, Fey V, Kaur A, **Knuuttila M,** Aho E, Oksala R, Westermarck J, Mäkelä S, Poutanen M, Aittokallio T. Optimized design and analysis of preclinical intervention studies *in vivo*. *Sci Rep.* (2016) 6:30723.

III. **Knuuttila M**, Mehmood A, Huhtaniemi R, Yatkin E, Häkkinen MR, Oksala R, Laajala TD, Ryberg H, Handelsman DJ, Aittokallio T, Auriola S, Ohlsson C, Laiho A, Elo LL, Sipilä P, Mäkelä S, Poutanen M. Antiandrogens reduce intratumoral androgen concentrations and induce androgen receptor expression in castration-resistant prostate cancer xenografts. *Am J Pathol.* (in press)

IV. **Knuuttila M**, Mehmood A, Mäki-Jouppila J, Ryberg H, Taimen P, Knaapila J, Boström PJ, Ohlsson C, Venäläinen MS, Laiho A, Elo LL, Sipilä P, Mäkelä S, Poutanen M. Distinct intratumoral androgen profiles in primary prostate cancer are linked to TMPRSS2-ERG gene fusion and altered intratumoral androgen metabolism. *Manuscript* .

1 INTRODUCTION

Prostate cancer is the most common type of cancer among males in developed Western countries and the second most frequently diagnosed cancer in men worldwide (Torre et al., 2015). For instance, more than 180,000 men are diagnosed with prostate cancer every year in the USA, representing approximately 21% of all new cancer cases (Siegel et al., 2016). However, prostate cancer is usually diagnosed in older men (>60 years), and although it is a life-threatening disease, it typically develops and progresses relative slowly. Despite the increasing incidence of prostate cancer, the mortality rates are declining in developed Western countries, particularly because of improvements in prostate cancer treatment and early detection.

The essential role of androgens in the development and progression of prostate cancer has been known since the 1940s. The effect of castration and the androgen sensitivity of prostate cancer were first demonstrated in 1941 by Huggins & Hodges (Huggins & Hodges, 1941), and ever since, the backbone of metastatic prostate cancer therapies has been the suppression of production and/or action of androgens. Although most patients respond to androgen deprivation therapy (ADT), in a significant number of patients the disease eventually progresses to a fatal stage, previously referred to as androgen-independent or hormone-refractory prostate cancer. However, studies performed over the last decades have elucidated that androgens are still one of the key factors driving the growth of prostate cancer after ADT (Labrie, 2011). Subsequently, the term "androgen-independent" has been replaced by the term “castration-resistant” as it more properly describes the biology of this state of disease (Scher et al., 2008).

Intratumoral androgen biosynthesis, which indicates that prostate tumors produce androgens by themselves, either from adrenal precursors or *de novo* from cholesterol, has been recently introduced as one of the mechanisms by which castration-resistant prostate cancer (CRPC) maintains tumor growth (Cai & Balk, 2011; Fiandalo et al., 2014; Zhang et al., 2016). The findings have led us to a new era of prostate cancer drug development focusing on the inhibition of intratumoral androgen biosynthesis. As a result, the first compound designed to suppress intratumoral androgen biosynthesis, abiraterone acetate, a CYP17A1 inhibitor, has been available as a post-chemotherapy option for CRPC patients since 2011, prolonging the overall survival among patients (de Bono et al., 2011). Despite the promising results obtained with abiraterone acetate, there are no curative therapy options available for CRPC. Therefore, new efforts of basic research, disease modeling and drug development for novel CRPC therapies aiming at efficient blockade of androgen biosynthesis are certainly needed.

2 REVIEW OF LITERATURE

2.1 Prostate cancer

2.1.1 Characteristics of prostate and prostate cancer

Prostate cancer is a malignancy developing in the prostate, which is an exocrine gland and part of the male reproductive system, located beneath the urinary bladder and surrounding the urethra. The primary function of the prostate is to produce prostatic secretions to protect and nourish sperm cells (Isaacs, 1983). The prostate gland secretes hormones, proteolytic enzymes and other proteins into the seminal fluid, which is produced mainly by the seminal vesicles (Aumüller & Seitz, 1990). The prostate consists of four different zones based on their anatomic location: the anterior zone, the central zone, the transition zone and the peripheral zone of the prostate gland. Partly because the peripheral zone is the largest zone of the prostate in terms of volume, approximately 70% of all prostate cancer tumors are found in the peripheral zone (McNeal et al., 1988).

The prostate epithelium is formed by secretory luminal cells, neuroendocrine cells and basal cells, which line the ducts of the branching glandular structures. The epithelium is surrounded by the fibromuscular stroma, containing smooth muscle tissue, fibroblasts, nerves, endothelial cells and lymphatic tissue (McNeal, 1988). The luminal cells grow in an androgen-dependent manner, whereas the growth of the basal cells lying beneath the luminal cell layer is not regulated by androgens. The vast majority of all prostate cancer tumors are classified as acinar adenocarcinomas of the prostate, arising from the luminal cells of the glandular epithelium of the prostate (Bostwick, 1989). The luminal cells are generally believed to be the origin of prostate tumors because prostate tumors typically express luminal cell markers such as cytokeratins and lack basal cell marker expression (Okada et al., 1992; Parsons et al., 2001). However, it is also possible that basal cells can initiate prostate cancer carcinogenesis, as presented in studies with genetically altered benign human basal cells grown in immunodeficient mice (Goldstein et al., 2010).

Histologically, prostatic intraepithelial neoplasia (PIN) lesions are considered to be precursors of prostate cancer, as high-grade PIN lesions in particular possess premalignant features (McNeal & Bostwick, 1986; Bostwick & Brawer, 1987). PIN is defined as cytologically atypical or dysplastic epithelial cells growing within the histologically benign epithelial glands and can be graded from mild, low-grade PIN to moderate or severe, high-grade PIN. High-grade PIN lesions are commonly found in the prostate of older men in four different histological

patterns: tufting, micropapillary, cribriform and flat patterns (Cheville et al., 1997). On the molecular level, high-grade PIN is characterized as an intermediate condition between the benign prostate epithelium and adenocarcinoma as it shares many genetic markers with prostate cancer (Brawer, 2005). However, the cells in high-grade PIN lesions are not capable of invading the surrounding tissues as cancer cells do. Nevertheless, studies suggest that most patients diagnosed with PIN lesions will develop adenocarcinoma within the following 10-year period (Bostwick, 1999).

2.1.2 Diagnosis of prostate cancer

In most cases, local prostate cancer causes no symptoms. Lower urinary tract symptoms in patients might be caused by non-malignant prostate diseases such as benign prostate hyperplasia (BPH) (Hollingsworth & Wilt, 2014). Thus, if symptoms occur, digital rectal examination together with serum prostate-specific antigen (PSA) testing is commonly recommended to evaluate the possibility of prostate cancer (Catalona et al., 1994). Nevertheless, in suspicious cases, a needle biopsy is necessary to confirm the presence of prostate cancer.

PSA testing became the main tool for prostate cancer diagnostics because of its convenience as a serum marker since its introduction in the late 1980s. As a result, the number of prostate cancer diagnoses in most Western countries has rapidly increased (Welch & Albertsen, 2009). PSA is an androgen-regulated serine protease secreted by the prostate epithelium to the seminal fluid. The concentrations of PSA in the prostate and seminal fluid are high, however, small amounts of PSA leak into the blood circulation. The disruption and dedifferentiation of the epithelium layer caused by adenocarcinoma also allows PSA to enter the blood circulation more efficiently, elevating serum PSA levels in men with prostate cancer (Balk et al., 2003). Although it is globally used for prostate cancer diagnostics, PSA is not a cancer-specific marker, as inflammation and BPH can elevate serum PSA levels as well (Stamey et al., 1987). Using serum PSA testing for screening to reduce prostate cancer mortality is considered controversial (Hayes & Barry, 2014). For population-based screening, PSA lacks specificity and sensitivity for early disease detection, leading to false-positive diagnoses and, thereby, unnecessary and harmful biopsies and treatments that outweight the possible benefits of PSA screening (Duffy, 2014). In addition to being a serum marker for detecting prostate cancer, PSA is widely used to monitor the responses of prostate cancer therapies.

The Gleason grading system is the most widely used grading system for prostate cancer, introduced 50 years ago by Donald F. Gleason (Gleason, 1966).

Commonly known as the “Gleason score”, the system is used to evaluate the histologic differentiation of prostate adenocarcinoma samples. Using five different grading patterns ranging from well-differentiated, uniformly shaped glandular structures to poorly differentiated structures, evaluated prostate cancer samples are given a score based on the most frequently observed histological patterns in a specimen (Humphrey, 2004). Despite certain limitations, to date, the Gleason grading system has proven to be the most reliable and reproducible method for predicting the clinical outcome of prostate cancer (Gordetsky & Espstein, 2016). Similarly to other solid tumor types, prostate adenocarcinomas are submitted to the TNM Classification of Malignant Tumors (TNM, tumor, lymph node, and metastasis) cancer staging system. Unlike the Gleason grading system developed for the histologic evaluation of prostate cancer cells, the TMN classification system is used to describe the stage of the disease, including the size of the tumor and the status of lymph nodes and metastases (Hoedemaeker et al., 2000).

2.1.3 Localized prostate cancer

Prostate cancer is a heterogeneous disease, thus, patients are treated with a wide variety of different therapies. In principle, active surveillance, surgical removal of the prostate (radical prostatectomy) or radiation therapy are the primary options for treating localized primary prostate cancer. Radical prostatectomy can also be combined with radiation therapy (Heidenreich et al., 2008). A combination of androgen deprivation and radiation therapy is commonly applied to patients with high-risk or locally advanced prostate cancer. The main parameters considered when choosing a treatment are the following: patient age, comorbidities, serum PSA, Gleason score, clinical tumor stage (TNM) and the amount of carcinoma in biopsies (Heidenreich et al., 2014).

Patients who have an organ-confined disease often undergo radical prostatectomy, and are operated using robotic-assisted laparoscopic radical prostatectomy (RALP), laparoscopic radical prostatectomy or traditional open radical prostatectomy. The studies conducted by the Scandinavian Prostate Cancer Group have demonstrated that for patients with palpable prostate tumors, radical prostatectomy is associated with decreased overall mortality (Bill-Axelson et al., 2011). On the other hand, active surveillance can be the most suitable option for patients with clinically insignificant low-risk prostate cancer. In active surveillance, patients will be monitored on a regular basis and only patients with clinically significant prostate cancer detected during the surveillance will be treated. Without compromising overall survival, the side

effects of unnecessary treatments can be avoided with active surveillance for the majority of patients with early-detected indolent prostate cancer (Parker, 2004). For patients with low-risk prostate cancer, the likelihood of dying of other causes is almost 20 times greater than that of dying of prostate cancer (Klotz et al., 2010). A recent prospective and randomized trial with 10-year follow-up demonstrated that active surveillance is as effective treatment option as radical prostatectomy and radiation therapy for localized prostate cancer in terms of survival (Hamdy et al., 2016). However, there is a lack of robust evidence for making treatment decisions, thus, clinicians must estimate the benefits and risks of active surveillance together with each patient (Simpkin et al., 2015).

2.1.4 Androgen deprivation therapy

Androgen deprivation therapy (ADT), also sometimes referred to as androgen ablation, has been the first-line therapy for advanced prostate cancer for many decades. Androgen deprivation can be achieved by castration, either by surgical removal of testes (orchidectomy) or by treating patients with gonadotropin-releasing hormone (GnRH) analogues (agonist or antagonist), to suppress the production of luteinizing hormone (LH), which further regulates the androgen production in gonads (Cooper & Page, 2014). Several studies have provided evidence of the efficacy of ADT for treating prostate cancer. First, the Veterans Administration Cooperative Urological Research Group demonstrated that ADT prevents metastatic disease progression but has no effect on survival rate among prostate cancer patients (Byar, 1973). Furthermore, combining ADT and radiation therapy increased overall survival in men with high-risk localized prostate cancer (D'Amico et al., 2008). Immediate ADT after radical prostatectomy reduced the risk of recurrence and improved survival in locally advanced prostate cancer patients (Messing et al., 1999; Jones et al., 2011). In general, patients treated with GnRH analogues generally show exceptional response rates to the therapy. Serum PSA levels can remain low or undetectable for months and years after the initiation of therapy, yet in many patients the disease progresses to castration-resistant prostate cancer over a period of approximately 18 months, with a median survival of 1 to 2 years (Ross et al., 2008, Figure 1).

In healthy men, medical castration can decrease testosterone levels in serum by up to 94%. Nevertheless, significant amounts of intraprostatic testosterone and dihydrotestosterone (DHT), approximately 20-30% of pre-castration levels, persist within the prostate, which are enough to maintain androgen receptor (AR) activation and androgen-regulated gene expression (Page et al., 2006). Similarly,

studies have verified that patients with recurrent tumors receiving ADT have sufficiently high intraprostatic androgen levels to activate AR (Titus et al., 2005a; Nishiyama et al., 2005). On the other hand, if men are treated with testosterone replacement therapy (TRT) because of hypogonadism, TRT typically normalizes serum androgen concentrations, while only modest changes in intraprostatic androgen levels are achieved (Marks et al., 2006; Thirumalai et al., 2016). Even exogenous DHT supplementation does not significantly alter intraprostatic levels of androgens (Page et al., 2011). Thus, although ADT reduces androgen levels in the circulation, prostate tumors can adapt to the altered androgen environment and eventually progress to the castration-resistant stage. In line with this observation, according to one of the few established mechanisms of CRPC progression, prostate tumors can evolve to castration resistance either by becoming hypersensitive to low levels of androgens or other steroidal ligands acting via AR, or by synthesizing androgens via the activation of intratumoral androgen biosynthesis (Penning, 2014).

2.1.5 Metastatic prostate cancer

Metastatic prostate cancer is the lethal form of prostate cancer. Suppression of testicular androgen production by ADT has been the backbone for treating metastatic prostate cancer since the 1940s, however, most patients eventually progress to metastatic castration-resistant prostate cancer (mCRPC). The field of metastatic prostate cancer therapies has developed rapidly in recent years, as new agents have entered the market, of which many are not targeting androgen action (Attard et al., 2016). Consequently, the emergence of these new drugs has improved the survival of metastatic prostate cancer patients (Tangen et al., 2012). As the number of prostate cancer patients has been estimated to keep increasing (Kvåle et al, 2007; Maddams et al., 2012), better therapies are obviously needed. In addition, the improved survival has increased non-cancer-related comorbidities among prostate cancer patients. ADT itself causes severe adverse effects, particularly the increased risk of cardiovascular complications (Tsai et al., 2007; Nguyen et al., 2015; Lester & Mason, 2015). Therefore, a major proportion of metastatic prostate cancer patients are not dying from prostate cancer.

Two chemotherapy agents, namely, docetaxel and cabazitaxel, have been demonstrated to improve survival in early-phase metastatic prostate cancer patients (Tannock et al., 2004; de Bono et al., 2010). Furthermore, docetaxel is used in a combination with ADT for metastatic hormone-sensitive prostate cancer (Sweeney et al., 2015). The bone metastases are the most commonly

found, in about 80% of all metastatic prostate cancer patients (Rigaud et al., 2002; Gandaglia et al., 2014). Thus, bone-targeting therapies, such as zoledronic acid and denosumab, are beneficial for treating complications caused by bone metastases, however, both drugs have showed no effect on improving the survival of patients (Attard et al., 2016). Radium-223 is an α-emitting bone-targeting radioisotope that mimics calcium. Thus, radium-223 is particularly used for treating patients with bone metastases, improving the overall survival of men with metastatic prostate cancer (Parker et al., 2013). Sipuleucel-T is an immune-based treatment form, which has shown a significant effect on survival of men with mCRPC (Graff & Chamberlain, 2015).

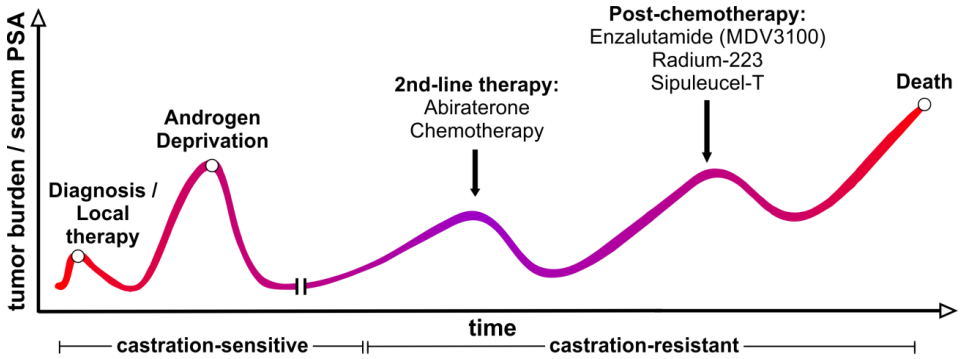

Figure 1. The typical treatment strategy for castration-resistant prostate cancer. The schematic timeline describes the different therapy options for treating prostate cancer that progress to the castration-resistant stage. Serum PSA measurements are commonly used to monitor the responses of prostate cancer therapies. If local therapy (radical prostatectomy and/or radiation therapy) fails or is not suitable, the patient is treated with androgen deprivation therapy (ADT), which is the first-line therapy for advanced prostate cancer patients. However, despite the initial response, in many patients the disease progresses to castration-resistant prostate cancer (CRPC). Usually, the second-line treatment option is chemotherapy (docetaxel or cabazitaxel), in particular for patients with metastatic castration-resistant prostate cancer. However, abiraterone acetate has been demonstrated to be beneficial prior to chemotherapy as well. Subsequently, the patients receive post-chemotherapy treatments. The patients are treated with either second-generation antiandrogens (enzalutamide) or other CRPC therapies (radium-223, sipuleucel-T). These agents prolong the overall survival of patients. Although many patients respond to post-chemotherapy treatments, none of these treatments are curative.

2.2 Androgen biosynthesis

2.2.1 Classical androgen production and regulation

Steroidogenesis is an endocrine process in which steroids are synthesized from cholesterol into various steroid hormones: glucocorticoids, mineralocorticoids and sex steroids; androgens, estrogens and progestogens, and secreted into the blood circulation to mediate their effects in target organs (Miller & Auchus, 2011). Steroid hormones regulate different physiological and developmental processes and share the same basic backbone structure composed of four interconnected carbon rings, mediating their effects via steroid hormone receptors and modulating the expression of specific target genes (Carson-Jurica et al., 1990; Figure 2). The most crucial steroid hormone receptors in endocrine action belong to the nuclear receptor subfamily 3. This subfamily includes estrogen receptors (ERα, ERβ), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR) and androgen receptor (AR) (Mangelsdorf et al., 1995).

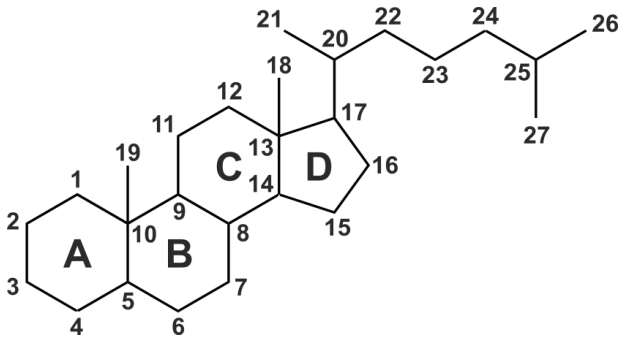

Figure 2. Steroid ring structure and atom numbering. The backbone structure of steroids is composed of four carbon rings (A-D) and numbered atoms (1-27).

Androgens are essential for the development, differentiation and maintenance of male sexual characteristics, and they regulate these same processes in the prostate (Cunha et al., 2004). Androgen biosynthesis and regulation in the testes is controlled by the hypothalamic-pituitary-gonadal (HPG) axis. Hypothalamus secretes GnRH, which stimulates the gonadotropic cells in the anterior pituitary to produce gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the blood circulation. Testosterone is produced in Leydig cells, where LH stimulates steroidogenesis, particularly for testosterone, by binding to the LH receptor (Miller & Auchus, 2011). The pulsatile manner of GnRH release is essential to stimulate LH secretion (Lincoln et al., 1986). Similarly to the HPG axis, the hypothalamic-pituitary-adrenal axis is an endocrine regulation system that controls steroid production in the adrenal glands.

2.2.2 Steroidogenesis for androgens

Steroidogenesis primarily occurs in endocrine organs, such as the testis, adrenal glands, ovary and placenta, but steroidogenic activity is also observed in other tissue types. Steroidogenesis is controlled by the expression and activity of various steroidogenic enzymes which can be principally divided into cytochrome P450 enzymes (CYP), which are expressed in mitochondria or the endoplasmic reticulum, and hydroxysteroid dehydrogenases (HSD) (Gonzalez, 1988; Miller, 2008). Hydroxysteroid dehydrogenases belong to either the short-chain dehydrogenase/reductase (SDR) or aldo-keto reductase (AKR) family (Miller & Auchus, 2011).

The first steps of steroidogenesis are identical regardless of the tissue where steroidogenesis occurs. All steroid hormones are derived from cholesterol, a precursor for steroid biosynthesis that is universally produced in all tissue types (Cerqueira et al., 2016). Cholesterol is transported into the mitochondria by steroidogenic acute regulatory protein (StAR). Subsequently, cholesterol is converted to pregnenolone by cholesterol side-chain cleavage enzyme P450scc (CYP11A1) in the mitochondria (Miller, 2002). Without CYP11A1 activity, steroidogenesis cannot be initiated. After this first rate-limiting step, pregnenolone is converted either by 3β-hydroxysteroid dehydrogenase/Δ5-4isomerase (HSD3B) to progesterone or by 17α-hydroxylase (CYP17A1) to 17α-OH-pregnenolone (Figure 3). Because HSD3B catalyzes the oxidative conversion of Δ5-hydroxysteroids to Δ4-ketosteroids, its expression is essential for the biosynthesis of androgens and estrogens, progesterone, glucocorticoids and mineralocorticoids (Lachance et al., 1992). In humans, two HSD3B isoforms have been reported. The biochemical properties of HSD3B1 and HSD3B2 are alike because of their nearly homologous amino acid sequences (Thomas et al., 2002). HSD3B1 expression is found in the placenta (Lorence et al., 1990), whereas HSD3B2 is mainly expressed in the gonads and adrenals (Rhéaume et al., 1991). Pregnenolone and progesterone are converted by CYP17A1, a dual-function enzyme that catalyzes sequential 17α-hydroxylase and 17,20-lyase reactions. 17α-hydroxylase catalyzes progesterone and pregnenolone to 17α-OH-progesterone and 17α-OH-pregnenolone, respectively. The 17α-hydroxylase reaction occurs at similar efficiency for both progesterone and pregnenolone, however, 17,20-lyase activity is very modest for 17α-OH-progesterone compared with that for 17α-OH-pregnenolone (Lee-Robichaud et al., 1995). Subsequently, 17,20-lyase converts the 21-carbon steroids to 19-carbon weak androgens, and its activity is observed in the human testes and adrenal glands in particular (Nakajin & Hall, 1981; Chung et al., 1987).

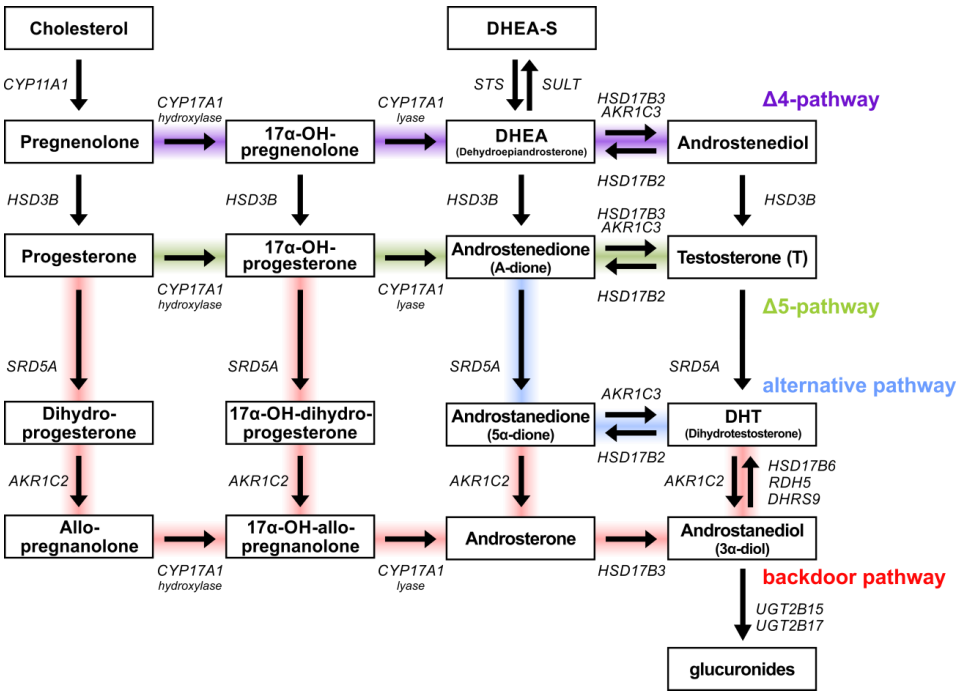

Figure 3. Androgen biosynthesis and metabolism. The schematic figure presents the main enzymatic steps of androgen biosynthesis from cholesterol to DHT via the Δ4-pathway, Δ5-pathway, alternative pathway and backdoor pathway.

The main circulating active androgen is testosterone (T), which is mainly produced in the testes, from which it is released into the blood circulation. The adrenal glands have a lesser role, contributing approximately 5-10% to total testosterone production. In humans, the testosterone circulating in the blood stream is bound to different serum proteins, namely, sex hormone-binding globulin (SHBG), corticosteroid-binding globulin and albumin, whereas only 2-3% of total T circulates as bioavailable free T (Taplin & Ho, 2011). After reaching the target tissue, testosterone is converted to dihydrotestosterone (DHT) in the prostate or in other target tissues, such as the seminal vesicles and epididymis, by 5α-reductases. To date, three different types of 5α-reductases have been reported. 5α-reductase type 1 (SRD5A1) is present in most tissues, whereas 5α-reductase type 2 (SRD5A2) is dominantly expressed in different genital tissues, including prostate tissue (Bartsch et al., 2000). The novel 5α-steroid reductase 3 (SRD5A3) was recently identified and observed to be overexpressed in hormone-refractory prostate cancer (Uemura et al., 2008). Although most of the testosterone produced in the testes is released into the circulation and not converted to DHT, minor amounts of DHT are synthesized in the testes (Hammond et al., 1977). Compared with testosterone, DHT has a higher affinity for AR and a longer biological half-life in the body, making it an up to 10-fold more potent androgen than testosterone (Grino et al., 1990). In

addition, because of effective 5α-reductase conversion from testosterone, DHT levels are up to 10-fold higher than testosterone levels in the prostate. However, it is worth mentioning that DHT levels are relatively low in serum, approximately 10% the level of testosterone, which is the most abundant active androgen in the circulation (Hay & Wass, 2009).

In addition to the extensive studies related to classical steroidogenesis in the testes and adrenals, the concept that prostate cancer cells are capable of producing sufficient amounts of active androgens has gained attention recently, although it was first hypothesized several decades ago by Geller et al. (Geller et al., 1979). Based on present knowledge, there are two plausible routes through which prostate tumors produce androgens, either by converting androgen precursors produced in adrenal glands or *de novo* (meaning "from the beginning") from cholesterol.

2.2.3 Adrenal androgen precursors

The adrenal glands in humans produce weak androgens often referred to as adrenal androgens, namely, androstenedione (A-dione), dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEA-S) (Adams, 1985), which all function as androgen precursors for more potent androgens such as testosterone and DHT. DHEA and DHEA-S are the most abundant steroids in the circulation. The biosynthesis of androstenedione and DHEA occurs in the zona reticularis, the innermost layer of the adrenal cortex, where DHEA is converted to DHEA-S and then transported to the peripheral tissues via blood circulation (Miller & Auchus, 2011). While the adrenals of humans and primates are capable of producing significant amounts of adrenal androgens, the plasma levels of DHEA-S and DHEA in rodents and domestic animals are considerable lower, indicating limited adrenal androgen biosynthesis in these species (Cutler et al., 1978). Consequently, it has been hypothesized that the adrenals of mouse and rat do not produce adrenal androgens because of the lack of 17α-hydroxylase (CYP17A1) enzyme (van Weerden et al., 1992). The main androgen precursor found in the circulation, DHEA-S, acts as a large reservoir for androgen metabolism as its concentrations in the circulation are up to 100-500 times higher than the serum levels of testosterone in healthy men (Bélanger et al., 1994). In the peripheral tissues, steroid sulfatase (STS) converts conjugated steroids such as DHEA-S to free DHEA, which can be further converted to more potent androgens in the prostate. Nevertheless, the cells in the zona reticularis express aldo-keto reductase type 3 (AKR1C3), which is capable of converting androstenedione to

testosterone. Thus, the human adrenal glands can also produce small yet significant amounts of testosterone (Nakamura et al., 2009).

Not long after the initial findings of surgical androgen deprivation by Huggins and Hodges (Huggins & Hodges, 1941), the role of the adrenals in prostate cancer was demonstrated by performing a bilateral adrenalectomy, which further decreased the low levels of androgens already reduced by orchidectomy (Huggins & Scott, 1945). In 1988, Klein et al. demonstrated that primary prostate cancer tumors and metastases have the same enzymatic machinery as normal prostatic tissue, indicating that prostate cancer tumors are at least capable of converting androstenedione to active androgens in the tissue (Klein et al., 1988). Several studies performed during the past decade have confirmed that adrenal precursors are converted to testosterone and DHT by prostate tumors. The expression of genes involved in androgen metabolism were shown to be increased in advanced prostate cancer samples, demonstrating that the conversion from adrenal androgens to testosterone can occur in prostate cells (Stanbrough et al., 2006). Studies conducted with prostate cancer cell lines VCaP and PC346C using pregnenolone and progesterone as substrates for androgen biosynthesis suggest that androgen biosynthesis from adrenal precursors is possible, whereas *de novo* synthesis from cholesterol would be unlikely to occur in the particular prostate tumors (Kumagai et al., 2013a). In addition, a gain-of-function mutation of HSD3B1 has been identified to increase the conversion of DHEA to DHT, transforming this normally rate-limiting step into a more efficient way of synthesizing DHT (Chang et al., 2013).

2.2.4 De novo androgen biosynthesis

Although there is clear evidence demonstrating that adrenal androgens can serve as a source for androgen metabolism to synthesize testosterone and DHT within prostate tumors, several studies have suggested that adrenal androgens are not necessary for intratumoral androgen biosynthesis. Based on studies involving LNCaP xenografts, the intratumoral concentrations of testosterone, DHT and progesterone were increased in castration-resistant LNCaP tumors, while the serum levels of steroids remained low after castration (Locke et al., 2008). Simultaneously, the expression of enzymes required for *de novo* androgen biosynthesis from cholesterol to DHT was detected in xenograft tumors at both mRNA and protein levels. Using radiolabeled acetic acid, a precursor of cholesterol, Locke et al. verified that the castration-resistant LNCaP xenografts can synthesize DHT *de novo* from cholesterol as a precursor (Locke et al., 2008).

Various studies have also utilized VCaP cells as a model cell line to study *de novo* androgen biosynthesis in prostate cancer. One of these studies suggests that AR activity in VCaP cells is both CYP17A1- and AKR1C3-dependent *in vitro* and *in vivo* (Cai et al., 2011). In addition, the authors demonstrated that VCaP cells in the castration-resistant stage are dependent on *de novo* androgen biosynthesis and can acquire resistance to CYP17A1 inhibitors even though abiraterone acetate decreases the intratumoral levels of testosterone and DHT in castration-resistant VCaP tumors. Using a similar type of xenograft model, Mostaghel et al. showed that circulating cholesterol levels are associated with intratumoral testosterone levels and tumor size, and correlate with CYP17A1 expression levels in these xenografts (Mostaghel et al., 2012). However, *in vitro* studies conducted with VCaP, LNCaP and LAPC4 prostate cancer cell lines claim that the androgen deprivation does not change the transcription of genes involved in cholesterol and androgen biosynthesis (Cheng et al., 2010). Nevertheless, this observation does not mean that these cells lines could not metabolize androgens *de novo* from cholesterol.

In addition to the studies performed using preclinical models, the expression of several steroidogenic enzymes, such as HSD3B1, HSD3B2, CYP17A1 and AKR1C3, has been confirmed to be up-regulated in prostate cancer metastases compared with primary tumors and benign prostate tissue, indicating that the metastases are capable of *de novo* androgen biosynthesis in castrated conditions (Montgomery et al., 2008). Furthermore, Sakai et al. demonstrated that a majority of clinical prostate cancer and benign hyperplasia samples express CYP17A1 enzyme, whereas its expression is detected in less than half of normal benign prostate specimens (Sakai et al., 2016).

The clinical significance of *de novo* androgen biosynthesis in CRPC remains controversial. Although studies have verified that at least some prostate cancer tumors are capable of *de novo* androgen biosynthesis, other studies suggest that the contribution of adrenal precursors converted to active androgens is a clinically more critical mechanism for castration-resistant disease progression and drug resistance. For instance, Hofland et al. found no clear evidence supporting *de novo* androgen biosynthesis in prostate cancer (Hofland et al., 2010). The majority of samples obtained from primary prostate cancer or CRPC patients showed either low or absent expression of steroidogenic enzymes involved in *de novo* androgen biosynthesis. In contrast, the expression of AKR1C3 and SRD5A1 was found to be abundant in the samples, supporting the concept that adrenal precursors are converted to testosterone and DHT in a clinical context.

2.2.5 Canonical, alternative and backdoor pathways

Regardless of whether intratumoral androgen biosynthesis occurs from adrenal androgen precursors or *de novo* from cholesterol, there are three steroidogenic pathways that are usually referred to as canonical/classical pathway, alternative pathway and "backdoor" pathway (Cai & Balk, 2011; Penning, 2014). In the following section, the characteristics of these three pathways are reviewed.

The canonical steroidogenesis pathway follows the well-characterized steroidogenesis in testes and adrenals, converting cholesterol to testosterone via both the Δ^5-pathway and Δ^4-pathway (Figure 3). It is proposed that prostate cancer cells metabolize androgens via the canonical pathway as testosterone is a direct precursor for DHT in the prostate (Penning, 2014). In the human testes, testosterone is preferably produced from pregnenolone to DHEA via the Δ^5-pathway (Flück et al., 2003). However, the catalytic activity of 17,20-lyase varies between different species, determining which pathway is more active. In contrast to humans, in rats, the Δ^4-pathway predominates testosterone production in the testes (Fevold et al., 1989). The 17,20-lyase reaction also requires cytochrome b5 activity, which enhances the 17,20-lyase reaction via the Δ^5-pathway by a factor of 50 compared with that via the Δ^4-pathway (Storbeck et al., 2013b; Miller & Auchus, 2011). Modest 17,20-lyase activity in the tissue means that the conversion to DHEA and A-dione is low, which in turn indicates that androgen biosynthesis moves towards the backdoor pathway.

Adrenal precursors DHEA and A-dione are converted to androstenediol and testosterone by reductive 17β-HSDs, respectively (Figure 3). 17β-HSD type 3 (HSD17B3) converts A-dione to T, but it is predominately expressed in Leydig cells in the testes (Geissler et al., 1994). Soon after the identification of HSD17B3, oxidative 17β-HSD type 2 (HSD17B2) and 17β-HSD type 4 (HSD17B4) were cloned and identified to inactivate testosterone and DHT by converting them to A-dione and 5α-androstanedione (5α-dione), respectively (Andresson et al., 1995; Adamski et al., 1995). Furthermore, HSD17B2 and HSD17B4 expression is present in the prostate and prostate cancer (Castagnetta et al., 1997). Koh et al. demonstrated that the expression levels of HSD17B3 were up-regulated in prostate cancer biopsies compared with those in benign prostate samples, whereas HSD17B2 expression levels were significantly lower in prostate cancer biopsies than in corresponding benign prostatic tissues (Koh et al., 2002). These observations suggest that prostate cancer tissue could convert A-dione to T more efficiently than the other way around.

AKR1C3, also known as 17β-HSD type 5, is one of the key enzymes involved in all three steroid pathways. AKR1C3 converts low affinity adrenal androgens to

more potent androgens, similarly to HSD17B3, and AKR1C3 is expressed in the normal prostate and the majority of prostate cancer samples (Penning et al., 2000; Nakamura et al., 2005). Although HSD17B3 is modestly expressed in the prostate, AKR1C3 expression clearly predominates, reaching levels up to 1000-10,000-fold higher in prostate cancer (Penning, 2014). Thus, the contribution of AKR1C3 to testosterone production in the prostate and prostate cancer has been suggested to be more critical than that of HSD17B3. In line with these findings, Fankhauser et al. demonstrated that AKR1C3 is overexpressed in hormone-refractory prostate cancer samples and hormone-refractory metastases, while they found no evidence supporting backdoor pathway activity that would significantly contribute to intratumoral androgen biosynthesis (Fankhauser et al., 2014). Furthermore, AKR1C3 has been identified as a novel androgen receptor selective coactivator promoting the growth of prostate cancer in xenograft models and CRPC samples (Yepuru et al., 2013).

Testosterone produced in the testes acts as an intermediate for DHT, which is metabolized from circulating testosterone in the prostate cells. The alternative and backdoor pathways, however, utilize metabolic routes that bypass testosterone in DHT biosynthesis (Figure 3). As a crucial part of both the alternative and backdoor pathways, 5α-reductase activity is required to convert A-dione to 5α-dione. SRD5A2 is considered the dominant 5α-reductase enzyme expressed in the normal prostate, whereas expression levels shift towards SRD5A1 in prostate cancer cell line models and recurrent prostate cancer tumors (Titus et al., 2005b; Stanbrough et al., 2006; Luu-The et al., 2008). In an experimental setup using prostate cancer cell lines and CRPC biopsies, the conversion of A-dione to 5α-dione occurred more rapidly than the conversion from A-dione to T, supporting the concept that the alternative pathway would be active in prostate cancer (Chang et al., 2011). It was also demonstrated that the growth of CRPC xenograft tumors is dependent on the conversion of A-dione to 5α-dione via the alternative pathway (Chang et al., 2011).

The activity of the backdoor pathway was first demonstrated in the tammar wallaby pouch testis during fetal development (Shaw et al., 2000; Wilson et al., 2003) and later in immature mouse testes (Mahendroo et al., 2004). Recent findings suggest that the backdoor pathway is also present in humans (Fukami et al., 2013). If the backdoor pathway is active, progesterone and 17α-OH-progesterone are converted by 5α-reductase to dihydroprogesterone and 17α-OH-dihydroprogesterone, respectively (Figure 3). Subsequently, dihydroprogesterone and 17α-OH-dihydroprogesterone are metabolized by 3α-HSD (AKR1C) to allopregnanolone and 17α-OH-allopregnanolone, respectively. These steroid metabolites are then converted by 17α-hydroxylase/17,20-lyase (CYP17A1) to androsterone, which is further metabolized to 3α-androstanediol (3α-diol). The

activation of the backdoor pathway in prostate cancer tumors has been suggested to be one of the mechanisms leading to castration resistance (Mostaghel & Nelson, 2008). Nevertheless, there is at least as much speculation as clear evidence demonstrating that intratumoral DHT levels found in CRPC tumors are actually synthesized via the backdoor pathway. Clinical studies demonstrated that the urinary metabolites of 17α-OH-allopregnanolone were increased in CRPC patients treated with abiraterone, a CYP17A1 inhibitor (Attard et al., 2012). 17α-OH-allopregnanolone metabolite levels also correlated with the levels of androsterone, a DHT precursor in the backdoor pathway. Likewise, androgen biosynthesis inhibitors finasteride and ketoconazole combined with AR antagonists increased the levels of 17α-OH-allopregnanolone in LNCaP cells *in vitro*, indicating activation of the backdoor pathway due to androgen biosynthesis inhibition (Locke et al., 2009). Furthermore, *in vitro* studies performed using the 22Rv1 prostate cancer cell line suggests that the backdoor pathway is the primary source of androgen biosynthesis in cells treated with 5α-reductase inhibitor dutasteride (Pham et al., 2014).

One of the key steps of DHT production in the backdoor pathway is the conversion of 3α-diol to DHT. The enzymes identified in converting 3α-diol to DHT are the following: 17β-hydroxysteroid dehydrogenase 6 (HSD17B6), also known as retinol dehydrogenase (RODH) and RODH-like 3α-HSD (RL-HSD), retinol dehydrogenase 5 (RDH5), 17β-hydroxysteroid dehydrogenase 10 (HSD17B10), short-chain dehydrogenase/reductase family member 9 (DHRS9) and retinol dehydrogenase 4 (RODH4) (Mohler et al., 2011b). The role of HSD17B6 in particular could be crucial in the backdoor pathway as it is predominantly expressed in both benign prostate cells and prostate cancer compared with other enzymes with 3α-HSD activity, leading to AR transactivation when 3α-diol is present (Bauman et al., 2006b; Mohler et al., 2011a). A member of the aldo-keto reductase family, AKR1C2, acts as a reductive 3α-HSD enzyme, converting DHT to 3α-diol (Penning et al., 2007). Consequently, the pre-receptor regulation of AR activation via the backdoor pathway is at least partly controlled by AKR1C2 (reductive 3α-HSD) and HSD17B6 (oxidative 3α-HSD). Interestingly, these two enzymes are not typically co-expressed in the same cell types because AKR1C2 and HSD17B6 are predominately expressed in epithelial and stromal cells, respectively (Bauman et al., 2006a; Penning et al., 2008).

Taking all these findings into account, it is evident that canonical, alternative and backdoor pathways can contribute to intratumoral androgen biosynthesis in prostate cancer. Nevertheless, further studies and more sophisticated preclinical models are needed to better understand the specific roles of these pathways during the progression of prostate cancer to castration resistance.

2.2.6 Androgen conjugation, transport and inactivation

Androgens can be inactivated and excreted through various cellular processes that regulate the bioavailability and elimination of numerous compounds. Phase II catabolism reactions such as glucuronidation and sulfation, which classically occur in the liver and kidney, are primarily responsible for conjugating androgens and other steroids (Bélanger et al., 2003). It was previously believed that phase II catabolism occurs only in tissues such as liver and kidney that have high catabolic activity. However, the expression of enzymes responsible for glucuronidation and sulfation has been found in different endocrine organs, including the prostate (Mueller et al., 2015). This observation has revolutionized the understanding of androgen metabolism and inactivation, emphasizing the fact that the regulation of androgen bioavailability and inactivation also occurs in androgen target tissues.

UDP-glucuronosyltransferases (UGTs) are conjugating enzymes that catalyze the glucuronidation of a vast number of xenobiotic and physiological compounds, such as drugs, steroids and bile acids. UGTs are most abundantly expressed in tissues such as the liver, kidney and skin because of their essential role in excretion (King et al., 2000). They utilize UDP-glucuronic acid (UDPGA) as a substrate for the formation of glucuronides by adding a polar glucuronosyl group from UDPGA to small hydrophobic molecules such as steroids that are subsequently excreted in urine and bile (Tukey & Strassburg, 2000). UGTs are divided into the UGT1 and UGT2 families according to their chromosomal localization and sequence homology (Mackenzie et al., 2005). Glucuronidation has been hypothesized to be a critical factor in the regulation of androgen action in prostate cancer. The enzymes UGT2B7, UGT2B15 and UGT2B17 in particular are expressed in androgen-sensitive tissues, similarly to enzymes in the prostate, and are mainly responsible for the local inactivation of androgens (Bélanger et al., 2003; Chouinard et al., 2007). These UGTs target either the 3-OH-group or 17-OH-group of androgens, including DHT and its metabolites androsterone and 3α-diol (Bélanger et al., 2003). UGT2B15 is only found in luminal cells of the prostate, whereas UGT2B17 is expressed in basal cells (Barbier et al., 2000). Interestingly, the expression levels of UGT2B15 and UGT2B17 have been demonstrated to be up-regulated in prostate cancer tumors after ADT (Grosse et al., 2013). Furthermore, antiandrogen treatment stimulated androgen glucuronidation in LNCaP cells, and the knockdown of UGT2B15 and UGT2B17 reduced anti-proliferative effects of antiandrogen treatment (Grosse et al., 2013).

In addition to glucuronidation, sulfation and desulfation regulated by sulfotransferases (SULTs) and steroid sulfatase (STS) are metabolic modification processes that are applied to androgen inactivation. Both of these modifications are needed in the hydrolysis and esterification of sulfate groups (Mueller et al., 2015). Among all cytoplasmic SULT family members, SULT1A1, SULT1E1, SULT2A1 and two isoforms of SULT2B1, SULT2B1a and SULT2B1b, have been identified to contribute to steroid sulfation (Hobkirk, 1985; Strott, 2002). SULT2A1 is the most crucial SULT related to androgen sulfation as it is predominantly expressed in the zona reticularis of human adrenal glands, converting DHEA to circulating DHEA-S, although DHEA can be sulfated by SULT1E1 or SULT2B as well (Rege et al., 2014). SULT2B1b has also been suggested to modulate AR signaling and growth of prostate cancer cells (Vickman et al., 2016).

In contrast to SULT2A1 and its role in DHEA sulfation, the primary function of STS is to activate sulfated steroids to free steroids (desulfation), mainly DHEA-S and estrogen sulfates to corresponding active steroids in endocrine tissues such as the prostate and ovaries (Mueller et al., 2015). Furthermore, STS is expressed in prostate cancer cell lines (Nakamura et al., 2006). The active uptake of DHEA-S into prostate cells is mainly controlled by OATP1A2 (organic anion transporting polypeptide 1A2) transport, and its expression has been demonstrated to be increased in several prostate cancer cell lines by androgen deprivation (Arakawa et al., 2012). Many other steroid transporters that belong to the SLCO and ABC gene families have been recently described to specifically transport steroids into prostate cells, elucidating the role of the active transportation and passive diffusion of steroids in intratumoral androgen biosynthesis (Cho et al., 2014). Thus, it appears likely that the increased uptake of DHEA-S and its desulfation to DHEA by STS in particular can contribute to the growth and progression of prostate cancer, especially for ADT-treated patients whose circulating T levels are low.

2.2.7 Novel androgenic ligands

Although T and DHT are the primary androgens that activate AR, many non-canonical androgens such as androgen precursors and other androgen metabolites are capable of AR binding. AR mutations broaden the ligand specificity of AR, causing promiscuous activation of AR by ligands such as glucocorticoids, estrogens, antiandrogens, pregnenolone and progesterone (Coutinho et al., 2016). In addition, a few novel androgenic ligands that can also notably activate the wild type AR have been recently identified.

Along with classical adrenal androgens A-dione, DHEA and DHEA-S, human adrenals produce 11β-hydroxyandrostenedione (11OHA4), which is converted from A-dione by CYP11B hydroxylation (Swart et al., 2013; Bloem et al., 2013). Although 11OHA4 itself has no significant androgenic activity (Bélanger et al., 1993), 11OHA4 can be metabolized in prostatic tissues by 5α-reductase, converting 11OHA4 to 5α-reduced metabolites that can activate AR similarly to DHT (Storbeck et al., 2013a). Adrenal glands produce significant quantities of 11OHA4, as its levels in the circulation are comparable to those of A-dione (Yokokawa et al., 2009). Based on current knowledge, 11OHA4 is most likely converted to 11β-ketoandrostenedione (11KA4) by 11β-HSD type 2 (HSD11B2) in androgen-responsive target tissues where HSD11B2 expression is abundant (Albiston et al., 1994). This suggestion is supported by the observation that 11KA4 levels in the adrenals are low (Rege et al., 2013). In addition, the conversion from 11OHA4 to 11KA4 has been demonstrated to occur in LNCaP cells, confirming the crucial role of HSD11B2 in this reaction (Swart et al., 2013). Taken together, 11OHA4 might serve as a precursor for novel androgenic ligands produced in prostate cancer, particularly when the intratumoral and serum levels of classical androgens T and DHT are reduced by ADT.

Studies have shown that 11OHA4 must be metabolized to 11-ketoandrogens to activate its androgenic properties. Storbeck et al. demonstrated that 11OHA4 can be converted to novel AR ligands, 11-ketotestosterone (11KT) and 11-ketodihydrotestosterone (11KDHT) (Storbeck et al., 2013a, Figure 4). These 11-ketoandrogens can be metabolized when the necessary enzymatic machinery is present, including 5α-reductase, 17β-HSD and 11β-HSD enzymes (Swart & Storbeck, 2015). 11KT and 11KDHT bind to the AR with potencies that are similar to those of T and DHT, respectively, and induce androgen-regulated gene expression changes and cell proliferation (Pretorius et al., 2016a). 11-ketoandrogens are not the only novel ligands that can activate the wild type AR. 11-deoxcorticosterone (DOC), a precursor of corticosterone, has been identified as a novel substrate for SRD5A1, and DOC and its 5α-reduced metabolite 5α-dihydrodeoxycorticosterone (5α-DH-DOC) have been demonstrated to promote cell proliferation through AR activation in prostate cancer cell lines (Uemura et al., 2010). Furthermore, 5α-DH-DOC was present in clinical tumor samples obtained from CRPC patients. These findings underline the observations that 11-ketoandrogens are derived from adrenal precursors, particularly from 11OHA4. However, a recent study demonstrated that 11KT is extensively produced in gonads, more specifically in Leydig cells in the testes and theca cells in the ovaries, thus, making 11KT a major androgen in humans (Imamichi et al., 2016). Marked 11KT and 11KDHT levels were also detected in prostate tissue samples and plasma obtained from prostate cancer patients (du Toit et al., 2016). Furthermore, plasma concentrations of 11OHA4, 11KA4 and 11KT were

remarkably higher in both 21-hydroxylase (CYP21A) deficient men and women, supporting the hypothesis that these novel androgenic ligands are not only synthesized in androgen target tissues but also in the adrenals or gonads (Turcu et al., 2016).

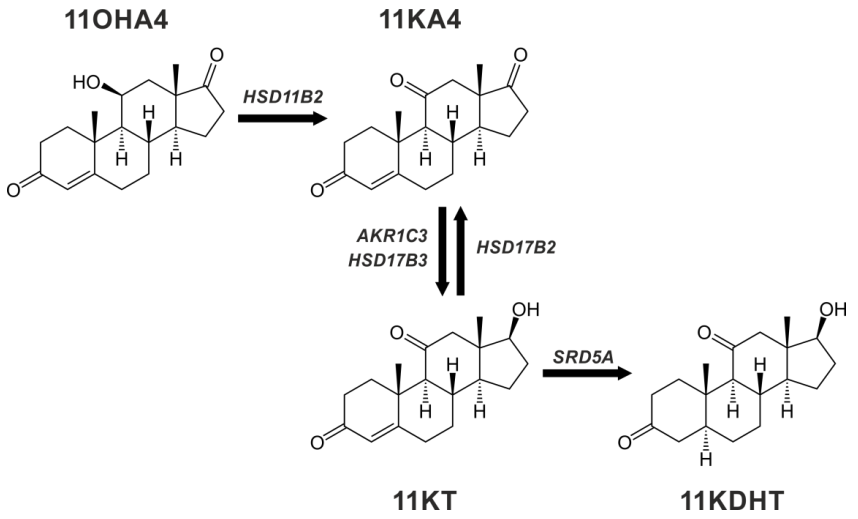

Figure 4. The biosynthesis of 11-ketoandrogens. 11β-hydroxyandrostenedione (11OHA4), which is mainly produced in the adrenals, is converted to 11β-ketoandrostenedione (11KA4) by 11β-HSD2 (HSD11B2) in the prostate. Similarly to androstenedione, 11KA4 is metabolized by 17β-HSB and 5α-reductases to more potent 11-ketoandrogens, 11KT (11-ketotestosterone) and 11KDHT (11-ketodihydro-testosterone). Both 11KT and 11KDHT activate AR as effectively as testosterone and DHT, respectively.

2.2.8 Preclinical intratumoral biosynthesis models for prostate cancer

Androgen biosynthesis is a complex endocrine process, elegantly regulated by multiple organs via hormone signaling. Thus, cell-based *in vitro* assays or even tissue cultures are not able to mimic the conditions needed for intratumoral androgen biosynthesis, particularly due to a lack of blood circulation carrying different androgen precursors and hormones secreted by endocrine organs such as the adrenals, pituitary and testes. Tissue samples obtained from prostate cancer patients are valuable for gene expression studies, for example. However, the tissue samples as such cannot be used to test and study the effects of novel CRPC drugs *in vivo*. To date, xenograft studies performed by grafting established prostate carcinoma cell lines to host species, usually to mice, have been the most convenient methods for modeling intratumoral androgen biosynthesis in prostate cancer.

One of the most recent prostate cancer cell lines utilized in both *in vitro* and *in vivo* studies is VCaP (Vertebral-Cancer of the Prostate), a prostate cancer cell line originally derived from a vertebral metastatic lesion from a patient with hormone refractory prostate cancer (Korenchuk et al., 2001). VCaP cells are androgen-dependent and express numerous clinical characteristics typical of prostate cancer, including PSA and AR, and when engrafted, they form tumors *in vivo* (Korenchuk et al., 2001). VCaP cells exhibit endogenous wild-type AR gene amplification, which results in high AR expression at both mRNA and protein levels (van Bokhoven et al., 2003).

Unlike the majority of prostate carcinoma cell lines such as PC-3, LNCaP and DU145, VCaP cells contain TMPRSS2-ETS fusions, including TMPRSS2-ERG fusion oncogene (Loberg et al., 2006). In addition, compared with LNCaP and C4-2 cell lines, for example, VCaP cells express high levels of steroidogenic enzymes AKR1C3 and HSD17B6 (Cai et al., 2011). Moreover, VCaP cells express steroidogenic enzymes CYP11A1, HSD3B2, HSD17B3 and SRD5A1, all crucial for steroid biosynthesis (Cai et al., 2011). As VCaP cells are suitable for xenograft studies, they can be used to model castration resistance *in vivo*. It has been demonstrated that VCaP cells grown in intact mice form tumors in an androgen-sensitive manner, however, if the mice are castrated, VCaP cells can retain the tumor growth independently of circulating androgens (Loberg et al., 2006). Overall, the aforementioned cellular and physiological properties of the VCaP cell line make it an excellent model for studying intratumoral androgen biosynthesis and androgen-dependent growth of prostate cancer.

2.3 Androgen receptor signaling

Several different mechanisms associated with androgen signaling have been shown to be involved in the development and progression of prostate cancer to castration resistance. Most of these mechanisms are related to the signaling of the androgen receptor (AR), a nuclear receptor mediating the biological effects of androgens. The mechanisms include, for example, AR gene amplification inducing an up-regulation of AR gene expression, different AR mutations that change the ligand specificity of the AR and the initiation of AR splice variant expression whose role in castration-resistant prostate cancer is still unclear (Yuan et al., 2013).

2.3.1 Structure and function of AR

The AR is encoded by the AR gene located on the X chromosome Xq11-12 and translated into a 110 kD protein composed of 919 amino acids (Lubahn et al., 1988; Trapman et al., 1988). The AR gene consists of 8 exons, encoding the AR polypeptide structure, composed of four domains: an N-terminal domain (NTD) encoded by exon 1, a DNA-binding domain (DBD) encoded by exons 2 and 3, a hinge region and a C-terminal ligand-binding domain (LBD) encoded by exons 4-8 (Jenster et al., 1992; Yong et al., 1998; Figure 5). The AR is a ligand-activated transcription factor that belongs to the nuclear receptor superfamily (Mangelsdorf et al., 1995). Responsible for both the development and maintenance of secondary male characteristics and normal male sexual differentiation, the AR takes part in the initiation and maintenance of spermatogenesis (Holdcraft & Braun, 2004). In the prostate epithelium, the AR is expressed in androgen-dependent luminal cells.

The inactive cytosolic AR is bound to chaperone proteins such as heat shock proteins. Upon ligand binding, the AR undergoes a conformational change, a dissociation of heat shock protein and the formation of an active AR homodimer (Brinkmann et al., 1989). The AR homodimer is transported to the nucleus, in where it activates the gene transcription by binding to DNA-binding sites known as androgen response elements (AREs) in the promoter and enhancer regions of AR target genes. In addition, the DNA-bound AR homodimer complex interacts with coregulatory proteins known as coactivators and corepressors that either enhance or reduce the transactivation of AR target genes, respectively (Heinlein & Chang, 2002). Eventually, the activation or repression of AR target genes leads to biological responses such as AR-mediated cell proliferation or survival.

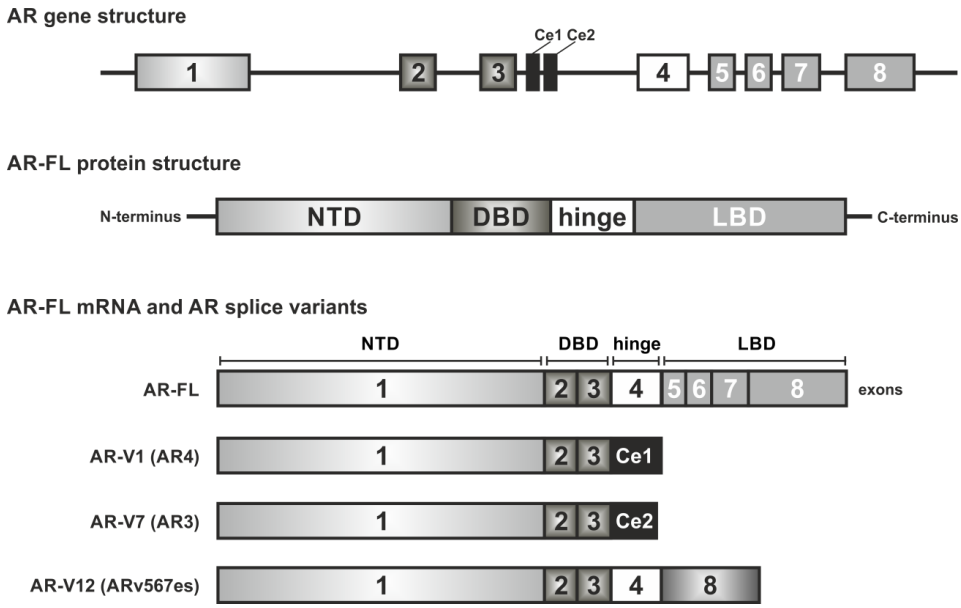

Figure 5. The structure of the AR and AR splice variants. The AR gene consists of 8 exons. The AR gene encodes the full-length AR (AR-FL) protein structure that contains N-terminal domain (NTD), DNA-binding domain (DBD), hinge region and ligand-binding domain (LBD). AR splice variants (AR-Vs) are truncated AR forms, of which many express cryptin exons (Ce). In addition to AR-Vs presented in this figure, several other cryptic exons and AR-Vs have been described.

2.3.2 AR overexpression

The up-regulation of AR signaling is one of the key mechanisms of the progression to castration resistance and drug resistance related to prostate cancer. By increasing the gene expression of AR, CRPC can compensate for low levels of androgens and retain the tumor growth (Waltering et al., 2009). AR overexpression is found in almost all recurrent prostate cancer tumors (Linja et al., 2001). The overexpression of the AR is frequently achieved by gene copy number alteration caused by DNA breaks, resulting in X-chromosome rearrangements and AR gene amplification (Visakorpi et al., 1995; Koivisto et al., 1997). AR amplification is rarely detected in primary and untreated prostate tumors, whereas in CRPC samples AR amplification is relatively common, particularly in patients with long exposure to ADT (Kumar et al., 2016; The Cancer Genome Atlas Research Network, 2015). In addition, recent studies have reported that the copy number gain of the AR in samples obtained from CRPC patients is associated with worse progression-free survival and drug resistance, with both abiraterone and enzalutamide therapies (Carreira et al., 2014; Wyatt et al., 2016).

2.3.3 AR mutations

AR mutations occurring in prostate cancer have been reported in various forms (Taplin et al., 1999; Wallén et al., 1999; Kumar et al., 2016). Approximately 20% of patients with advanced metastatic prostate tumors have been reported with AR mutations, however, no AR mutations have been found in patients with primary prostate cancer, suggesting that AR mutations play a minor role in the initial carcinogenesis of prostate cancer (Marcelli et al., 2000). Out of more than 150 different AR mutations identified in prostate cancer samples, in most cases, the mutations are located in the ligand-binding domain of the AR (Gottlieb et al., 2012). The somatic mutations leading to amino acid substitutions change the size of the ligand-binding pocket, allowing alternative ligands to bind to the AR. Many of these mutations are gain-of-function mutations, allowing the AR to be activated by non-androgenic steroid ligands present in the body, such as progesterone and estrogens (Taplin et al., 1995). The first identified AR-LBD mutation (T877A) in prostate cancer, originally found in the LNCaP cell line, causes a substitution of alanine for threonine at position 877 of AR protein (Veldscholte et al., 1990). In addition to androgens, the T887A mutant can be activated by progestogens, estrogens and antiandrogens such as flutamide (Veldscholte et al., 1990; Berrevoets et al., 1993). The T887A mutant is frequently found in patients treated with flutamide, suggesting that AR mutations occur in response to selective pressure driven by antiandrogen treatment (Taplin et al., 1999). In line with this finding, the recent identification of AR mutation F876L confirmed that CRPC tumors can gain drug resistance to antiandrogen treatment (enzalutamide) through AR mutations by an antagonist-to-agonist switch (Korpal et al., 2013).

2.3.4 AR splice variants

Great interest in different AR splice variants (AR-Vs) emerged shortly after they were detected in clinical prostate cancer samples, prostate cancer cell lines and xenografts (Guo et al., 2009; Hu et al., 2009; Dehm et al., 2008; Sun et al., 2010; Watson et al., 2010). Compared with the full-length AR (AR-FL), AR-Vs partly or completely lack the LBD caused by C-terminal deletion, leading to constitutive activity of the AR, as demonstrated in functional studies (Jenster et al., 1991; Simental et al., 1991). The structural changes of the truncated AR forms are achieved either by introducing cryptic exons or exon skipping (Coutinho et al., 2016; Figure 5). Because of a lack of functional AR-LBD, the truncated AR variants have been demonstrated to be active even in the absence of androgens, increasing androgen-regulated gene transcription ligand-

independently (Nyquist et al., 2013). These findings suggest that AR-Vs might play an important role in the progression of CRPC. Despite numerous functional studies, the significance of AR-Vs in CRPC remains unclear.

Although AR-Vs have been detected in normal prostate tissue, the highest AR splice variant expression levels have been found in CRPC samples (Guo et al., 2009). The most frequently found AR splice variant AR-V7, also known as AR3, has been shown to be up-regulated during prostate cancer progression and associated with disease recurrence after radical prostatectomy (Guo et al., 2009). Th expression levels of AR-V1, AR-V7 and AR-V567es, found in bone metastases obtained from CRPC patients, were significantly increased in metastases compared with primary tumors and correlated with poor patient prognosis with shortened survival (Hörnberg et al., 2011). The AR splice variants were also detectable in soft tissue metastases such as those in the liver and lymph nodes (Zhang et al., 2011).

Several mechanisms have been suggested to explain alternative AR splicing events, including alternative transcription start sites, alternative translation start codons and premature stop codons caused by mutations (Guo & Qiu, 2011). Because truncated AR-Vs are confirmed to be constitutively active, the question is raised regarding whether AR-Vs could be one of the mechanisms of resistance to ADT and novel CRPC therapies. The AR-V7 and AR-V1 splice variants in particular are frequently expressed in clinical CRPC samples (Hu et al., 2009). AR-V7 has been detected in circulating tumor cells from CRPC patients, in addition, detectable levels of AR-V7 are associated with resistance to both enzalutamide and abiraterone treatments (Antonarakis et al., 2014).

2.3.5 AR target genes

The androgen receptor is a transcription factor regulating the expression of hundreds of different genes known as AR target genes. The AR regulates the proliferation, survival and differentiation of prostate epithelial cells as well as the secretory functions of these cells in prostate. For instance, the well-characterized canonical androgen-regulated genes encode proteins such as kallikreins KLK2 and KLK3 (PSA) as well as prostatic acid phosphatase, which are the most abundant secretory proteins found in seminal fluid (Nelson et al., 1998). In addition to the well-known androgen-regulated genes, a vast spectrum of different AR target genes has been identified using transcriptome analyses such as microarrays and next-generation sequencing over the past decades (Nelson et al., 2002; Ngan et al., 2009; Jin et al., 2013).

Once the AR splice variants were first identified in CRPC, it was demonstrated that they can activate canonical androgen-regulated genes such as KLK3, FKBP5, NKX3.1 and TMPRSS2 even in the absence of AR-FL (Dehm et al., 2008; Guo et al., 2009; Hu et al., 2009). In this manner, AR-Vs could substitute for AR-FL-mediated signaling and modulate the same set of AR target genes. Based on recent findings, AR-Vs are also able to regulate an alternative gene expression signature different from that of AR-FL, however, only a few genes have been identified consistently, either up-regulated or down-regulated, by different research groups (Lu et al., 2015b). By using distinct transcriptional programs, AR-V can mediate AR signaling independently of ligand-dependent AR-FL, consequently providing resistance to AR-targeted CRPC therapies (Hu et al., 2012; Li et al., 2013).

2.3.6 TMPRSS2-ETS gene fusions

Similarly to other androgen-regulated genes, transmembrane protease serine 2 (TMPRSS2), an enzyme expressed in normal and malignant prostate epithelium, is androgen-regulated by the AREs in its promoter region (Lin et al., 1999). TMPRSS2 can be fused with ETS (erythroblast transformation-specific) family members and form TMPRSS2-ETS fusions. The ETS transcription factor family consists of 27 genes contributing to various biological processes such as apoptosis, cell proliferation, angiogenesis, differentiation and invasiveness (Hessels & Schalken, 2013). TMPRSS2-ETS fusions lead to the overexpression of ETS genes that are not normally expressed in the prostate, activating oncogenic signaling driven by androgen-regulated TMPRSS2.

One of most studied ETS family members, v-ets avian erythroblastosis virus E26 oncogene homolog ERG (ETS related gene), was discovered in 1987 (Reddy et al., 1987). Its role as an oncogene in prostate cancer was described by Tomlins et al. relatively recently in 2005 (Tomlins et al., 2005). In addition to ERG, other common ETS family members that fuse with TMPRSS2 and are present in prostate cancer are ETV1, ETV4 and EVT5 (Tomlins et al., 2005; Tomlins et al., 2006; Helgeson et al., 2008). Compared with other ETS family genes, TMPRSS2-ERG fusions are the most common type of gene fusions occurring in prostate cancer, present in more than half of prostate cancer patients (Tomlins et al., 2005; Pettersson et al., 2012). TMPRSS2 and ERG loci are located at the same chromosome region 21q22.2-q22, where interstitial deletions or smaller microdeletions can cause rearrangements, bringing the gene loci together (Iljin et al., 2006; Hermans et al., 2006). As an early clonal event in prostate cancer, TMPRSS2-ETS fusions are commonly found in localized primary tumors and

CRPC metastases, however, they are rarely detectable in benign prostate tissue (Tomlins et al., 2005; Clark et al., 2007; Perner et al., 2007). Due to the high prevalence of TMPRSS2-ERG fusions in prostate cancer patients, TMPRSS2-ERG has been evaluated as a promising biomarker for predicting the outcome of prostate cancer patients because TMPRSS2-ERG fusion transcripts can be detected in the urine of prostate cancer patients (Laxman et al., 2006; Rostad et al., 2009). The detection of TMPRSS2-ERG fusion transcripts in the urine of prostate cancer patients has been associated with a less favorable clinical outcome (Rostad et al., 2009).

It is not completely clear how ETS gene rearrangements contribute to the development and progression of prostate cancer. For instance, ERG overexpression can induce malignant transformations of the prostate epithelium and promote cell invasion (Klezovitch et al., 2008). On the other hand, studies using a transgenic mouse model have shown that although ERG induces a neoplastic phenotype in mouse prostate, ERG overexpression is not sufficient to transform benign prostate cells into malignant tumor cells without other molecular alterations (Tomlins et al., 2008; Zong et al., 2009). Studies have associated ERG rearrangements with PTEN (phosphatase and tensin homolog) loss in prostate cancer, suggesting that these two events play a co-operative role (Han et al., 2009). PTEN is a tumor suppressor inhibiting oncogenic PI3K/AKT signaling (Salmena et al., 2008). Transgenic mouse model studies demonstrated that ERG overexpression together with PTEN loss leads to acceleration of the development of prostate cancer in the mouse prostate (Carver et al., 2009; Chen et al., 2013). Furthermore, AR signaling and PI3K/AKT signaling, which involves PTEN, have been reported to cross-regulate each other in prostate cancer by a reciprocal feedback mechanism (Lin et al., 2004; Carver et al., 2011).

Some studies suggest that prostate cancer tumors with TMPRSS2-ERG fusions have an aggressive phenotype, likely due to high ERG expression (Demichelis et al., 2007; Yoshimoto et al., 2008). Patients presenting with TMPRSS2-ERG fusions have also been associated with a favorable prognosis as the fusion-positive patients have statistically significantly longer progression-free survival after prostatectomy compared with fusion-negative patients (Saramäki et al., 2008). Despite the numerous studies that have associated TMPRSS2-ERG fusions with clinical outcomes, there is no consensus for clear conclusions (Barbieri & Rubin, 2015).

2.4 Therapies targeting androgen signaling in prostate cancer

A few major breakthroughs in the development of new therapeutic compounds for CRPC have taken place throughout the past decade. Some of the recently approved compounds for treating CRPC target androgen action, either by inhibiting androgen biosynthesis and/or by blocking the activity of the AR (Figure 6). Although the AR has been undoubtedly the most important drug target for CRPC therapies for decades, there are other potential ways to modulate androgen action.

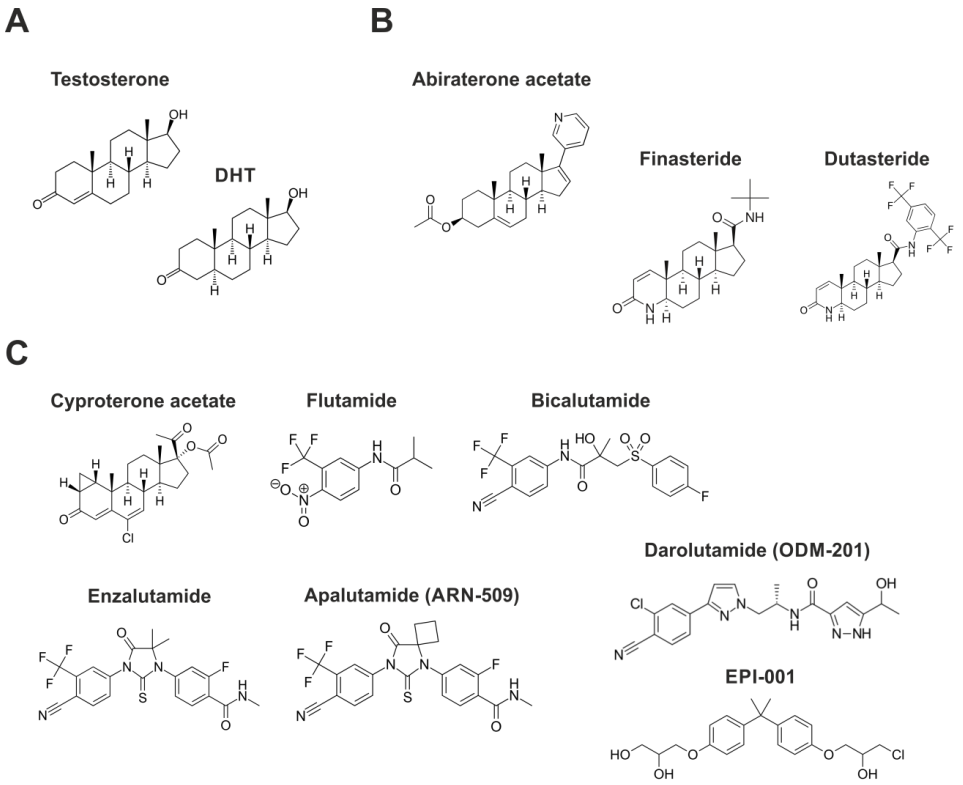

Figure 6. The chemical structures of androgens (A), androgen biosynthesis inhibitors (B), and AR antagonists (C). Testosterone and DHT are the primary androgens that activate AR. Abiraterone acetate is a CYP17A1 inhibitor, and currently the only androgen biosynthesis inhibitor approved for treating CRPC patients, whereas 5α-reductase inhibitors, dutasteride and finasteride, are given to patients suffering from benign prostatic hyperplasia. AR antagonists, also known as antiandrogens, presented in the figure are either been previously used, currently in use or in clinical trials for treating CRPC.

2.4.1 Antiandrogens

Antiandrogens, also known as AR antagonists, block the effects of androgens by directly binding to the AR. Traditionally, they have targeted the AR-LBD, being classified as steroidal antiandrogens or non-steroidal antiandrogens (NSAAs). Cyproterone acetate was the first antiandrogen developed in the early 1960s, followed by other steroidal antiandrogens such as megestrol acetate and medroxyprogesterone acetate (Neumann, 1983). In addition to demonstrated AR antagonism, cyproterone acetate inhibits gonadotropin secretion, leading to reduced testosterone and LH levels (Haendler & Cleve, 2012). Despite the potent antiandrogenic effects, the severe side effects and interactions with other steroid receptors, particularly PR and GR, have limited the clinical use of these antiandrogens in treating prostate cancer (Poyet & Labrie, 1985; Goldenberg & Bruchovsky, 1991). NSAAs were introduced in 1989, when flutamide was approved for treating advanced prostate cancer (Helsen et al., 2014). Later, bicalutamide and nilutamide were introduced, sharing a non-steroidal basic structure similar to that of flutamide. These antiandrogens have been used in combination with GnRH agonist/antagonist to achieve "complete" androgen blockade with ADT. However, there is limited evidence demostrating the benefits of using NSAAs for treating advanced prostate cancer as a monotherapy. Moreover, antiandrogens such as bicalutamide possess AR agonistic effects against prostate cancer cells (Culig et al., 1999). Thus, they are considered an unfavorable CRPC therapy option.

Enzalutamide is the first second-generation antiandrogen approved by the Food and Drug Administration for treating CRPC. The antiandrogen has several characteristics that make it superior to first-generation antiandrogens such as bicalutamide. First, enzalutamide binds to the AR with greater affinity, blocking the AR more effeciently, in addition, it impairs DNA binding to AREs and reduces the nuclear translocation of AR (Tran et al., 2009). ARN-509, a derivate of enzalutamide, has also been reported to possess antiandrogenic potency comparable to that of enzalutamide (Clegg et al., 2012). A recent clinical phase 2 study demonstrated that ARN-509, also now known as apalutamide, is safe and exhibits antitumor activity in patients with non-metastatic CRPC (Smith et al., 2016). In addition to these previously described second-generation antiandrogens, ODM-201 (darolutamide) was recently proposed as a new drug candidate for CRPC after successful phase 1/2 trials (Fizazi et al., 2014). Currently in a phase 3 trial, ODM-201 is also capable of blocking several AR mutants such as F876L unlike enzalutamide and ARN-509 (Moilanen et al., 2015).

Partly due to the discovery of AR splice variants and their role in CRPC, an interest in developing antiandrogens that do not target the LBD of the AR has emerged. As a minor breakthrough, EPI-001, a bisphenol A diglicycyl ether derivative, has been identified as a novel antiandrogen that binds covalently to the NTD of the AR. EPI-001 blocks protein-protein interactions that are required for transcriptional activity of full-length AR and possibly for AR splice variants (Andersen et al., 2010). EPI-001 is also a selective modulator of peroxisome proliferator-activated receptor gamma (Brand et al., 2015). Its successor EPI-506 is currently in clinical trials.

2.4.2 Androgen biosynthesis inhibitors

CYP17A1 has become the primary target enzyme for the development of novel therapies inhibiting androgen biosynthesis for its central role in steroidogenesis. Although many compounds have been demonstrated to inhibit CYP17A1, they also tend to inhibit other CYP enzymes in a non-specific manner. For example, ketoconazole is a non-specific weak CYP17A1 inhibitor that has been reported to inhibit androgen biosynthesis *in vivo* (Cai et al., 2011). Ketoconazole suppresses the residual adrenal androgens in CRPC patients, however, because of its limited efficacy and lack of specificity, which produce undesirable side effects, it is not suitable for treating prostate cancer (Bird & Abbott, 2016). Thus, remarkable efforts are required to develop specific CYP17A1 inhibitors to block androgen biosynthesis.

Abiraterone acetate, a steroidal CYP17A1 inhibitor, is currently the first and only androgen biosynthesis inhibitor used in clinical practice to treat patients with CRPC. Abiraterone acetate is a prodrug of abiraterone, which inhibits intratumoral androgen biosynthesis via irreversibly inhibiting both 17α-hydroxylase and 17,20-lyase activity of CYP17A1, resulting in an up to 75% decrease in serum T levels in castrated and non-castrated patients (O'Donnell et al., 2004). Due to the possible mineralocorticoid excess induced by CYP17A1 inhibition, abiraterone is administered in combination with prednisone or other glucocorticoids to avoid adrenal insufficiency causing severe hypertension and hypokalemia (Attard et al., 2012). Abiraterone acetate was shown to extend the overall survival of metastatic CRPC patients who had previously received chemotherapy for 3-4 months (de Bono et al., 2011). Treating mCRPC patients with abiraterone without previous chemotherapy has also been demonstrated to be beneficial by improving radiographic progression-free and overall survival and delaying the clinical decline and initiation of chemotherapy (Ryan et al., 2013). Furthermore, abiraterone acetate exhibited had antitumor activity in

CRPC patients who had been previously treated with ketoconazole (Ryan et al., 2010). However, although abiraterone acetate markedly reduced adrenal androgen concentrations in the serum of patients, DHEA-S levels still persisted up to 20 µg/dl in the circulation, possible serving as a depot for intratumoral conversion to testosterone and DHT in prostate tumors (Tamae et al., 2015). Interestingly, a novel abiraterone metabolite was recently identified by Li et al., who demonstrated that abiraterone is metabolized by HSD3B1 to its Δ^4-3-ketosteroid metabolite, also known as Δ^4-abiraterone (D4A) (Li et al., 2015). D4A inhibits CYP17A1, HSD3B1 and SRD5A enzyme activity and blocks the AR, suggesting an additional mechanism by which abiraterone treatment extends the overall survival of CRPC patients (Li et al., 2015).

Because antiandrogen treatment has been the cornerstone of CRPC therapies for decades, an interest in combining AR blockade with androgen biosynthesis inhibition has emerged. It has also been hypothesized that prednisone could activate AR and contribute to resistance to abiraterone in CRPC patients, which supports the rationale behind combined AR blockade and CYP17A1 inhibition (Richards et al., 2012). Several novel compounds have been reported to have such properties *in vitro*, and some of them have already entered clinical studies. For example, galeterone (TOK-001) is a novel steroidal antiandrogen demonstrated to act as a CYP17A1 inhibitor and AR antagonist (Njar & Brodie et al., 2015). Galeterone also induced proteasomal degradation of AR-FL and AR-V7 in CRPC xenografts (Kwegyir-Afful et al., 2015).

Although most novel androgen biosynthesis inhibitors target CYP17A1, there are other plausible ways to inhibit intratumoral androgen biosynthesis. AKR1C3 is another potential target enzyme for inhibiting androgen biosynthesis as it catalyzes the reduction of androstenedione to testosterone in the prostate, thus, regulating AR activity and ligand availability on the pre-receptor level. Different compounds and drugs are able to inhibit the activity of AKR1C3, including many non-steroidal anti-inflammatory drugs, flavonoids, steroid analogues and benzodiazepines (Byrns et al., 2011). However, the development of selective AKR1C3 inhibitors is challenging because the AKR1C3 sequence is >86% identical to other AKR1C family members AKR1C2 and AKR1C1, which both deactivate DHT in the prostate (Penning et al., 2000). Nevertheless, certain compounds, such as indomethacin, inhibit AKR1C3 activity with strong selectivity over AKR1C1 and AKR1C2 (Bauman et al., 2005). Furthermore, indomethacin has been demonstrated to block cell proliferation *in vivo* via AKR1C3 inhibition (Cai et al., 2011).

5α-reductase inhibitors, namely, dutasteride and finasteride, are commonly used to reduce the symptoms caused by benign prostatic hyperplasia (Bartsch et al., 2000). Finasteride inhibits specifically 5α-reductase type 1 (SRD5A1), whereas dutasteride is a dual inhibitor that effectively inhibits both SRD5A1 and SRD5A2 enzymes (Clark et al., 2004). Collective evidence shows that 5α-reductase inhibitors may slow down the progression of prostate cancer because they inhibit the conversion of testosterone to DHT (Cha & Shariat, 2011). However, the use of 5α-reductase inhibitors in prostate cancer prevention remains controversial. Based on two large randomized trials, 5α-reductase inhibitors decrease the incidence of prostate cancer but also increase the risk of high-grade prostate cancer tumors, however, these findings are debatable (Lacy & Kyprianou, 2014).

2.5 Preclinical animal models for prostate cancer

Prostate cancer mouse models, either human prostate cancer xenograft mouse models or transgenic mouse models, are essential tools for drug discovery as well as for understanding the development and progression of prostate cancer (Wu et al., 2013; Ittmann et al., 2013; Valkenburg & Pienta, 2015). Transgenic mouse models are useful models for studying the complex mechanisms of prostate tumorigenesis and progression (Abdulkadir & Kim, 2005; Pienta et al., 2008). The drug development of novel prostate cancer therapies relies greatly on animal experimentation, particularly on xenograft models which are cost-effective and practical models for studying the effects of drug candidates *in vivo* (Cheon & Orsulic, 2011; Jung, 2014).

2.5.1 Human prostate cancer xenograft models

Over the past decades, several human prostate cancer xenograft models have been generated based on established prostate cancer cells lines (van Weerden et al., 1996, van Bokhoven et al., 2003) In human prostate cancer xenograft models, human prostate cancer cells or fresh prostate cancer tissue samples are transplanted to host animals, typically to immunodeficient mice. The most common types of xenograft models for cancer research are subcutaneous and orthotopic models, in which human cancer cells are transplanted subcutaneously into the flank/back of mouse or into the original site of engrafted material, respectively (Jung, 2014). Subcutaneous xenograft models are more popular than orthotopic models as they are easy to perform and the tumor growth can be monitor by palpation (van Weerden et al., 2009; Wu et al., 2013). Conventional

imaging techniques such as ultrasonography, magnetic resonance imaging and computerized tomography can be used to monitor the tumor growth in orthotopic xenografts, however, optical imaging using cancer cell lines that express fluorescence or luciferase reporters can be utilized in their place (Frangioni, 2003; Hsu et al., 2006). Despite the technical advantages over orthotopic models, subcutaneous xenograft models lack several crucial biological features such as the capability to metastasize (Kerbel, 2003). In contrast, orthotopic models provide a better platform for epithelial-stromal interactions in an authentic microenvironment, which may play an essential role in the responses of drug interventions and endogenous steroid ligands (Talmadge et al., 2007).

Recently, patient-derived xenografts (PDXs) models have gained popularity in preclinical studies. In PDX models, cancerous cells or tissue material are collected from patients and directly transplanted into immunodeficient mice to fully resemble the characteristics of a particular tumor type (Inoue et al., 2017; Lai et al., 2017). PDX models have been suggested to provide better translation to clinical trials and could serve as valuable tools for optimizing personalized precision medicine (Gao et al., 2015).

2.5.2 Limitations of preclinical animal models

Despite the usefulness of preclinical animal models, the reproducibility and translatability of preclinical animal models should be improved to better facilitate drug development. The issues related to reproducibility and translatability are considered to be partially due to insufficient statistical and experimental design in preclinical animal models (Begley & Ellis, 2012; Freedman et al., 2015). To overcome these problems, it has been suggested that preclinical animal model experimentation should involve practices similar to those typically used in clinical trials (Muhlhausler et al., 2013). The main concerns that require improvement are the lack of randomization and blinded allocation of intervention groups and inadequate power calculations for estimating sufficient sample size for *in vivo* studies, all of which are standard requirements for clinical trials (Henderson et al., 2013). In addition, differences in animal baseline characteristics, such as gender, age, housing, body weight and genetic background, should be properly taken into account when conducting preclinical studies as they might contribute to confounding variability, for example, between the intervention groups (Hildebrand et al., 2013; Perrin, 2014). Although guidelines for standardizing preclinical animal studies have been established and are widely acknowledged, a recent literature review revealed that the vast majority of published animal studies lack the proper randomization of animals

and the estimation of statistically sufficient study size for detecting the distinct effects of the interventions (Baker et al., 2014). As a result, the lack of standardization in preclinical studies is likely one of the reasons why drug candidates frequently fail in clinical human trials despite being effective in animals (Kerbel, 2003; Perrin, 2014; Valkenburg & Pienta, 2015). Therefore, preclinical animal models that are standardized and optimized for drug development and better mimic the growth and progression of human prostate cancer are definitely needed.

3 AIMS OF THE STUDY

The progression of prostate cancer to castration resistance remains a significant clinical problem as the number of CRPC patients is constantly rising. To date, there are no curative therapies for CRPC. The drug development of novel CRPC therapies relies principally on the modulation of androgen action and biosynthesis, thus, understanding the mechanisms modulating intratumoral androgen biosynthesis is crucial. In order to develop and test novel CRPC therapies, more sophisticated preclinical animal models are urgently needed.

The primary objective of this doctoral study was to investigate intratumoral androgen biosynthesis in prostate cancer.

The specific aims of the study were:

- To establish a prostate cancer xenograft model suitable for studying novel CRPC therapies and intratumoral androgen biosynthesis *in vivo*
- To identify the mechanisms of intratumoral androgen biosynthesis *in vivo*
- To optimize the experimental design and statistical analyses for preclinical xenograft models
- To assess the effects of novel antiandrogens on androgen signaling and intratumoral androgen biosynthesis and metabolism
- To measure and evaluate the concentrations of androgens and the expression of androgen-metabolizing enzymes in cancerous and benign prostate tissue obtained from patients with primary prostate cancer

4 MATERIALS AND METHODS

4.1 Cell culture (I,III)

VCaP cells were purchased from ATCC (the American Type Culture Collection, Manassas, VA) and cultured on Corning CellBind® flasks (Corning Life Sciences, Tewksbury, MA) in phenol red-free RPMI-1640 (Sigma-Aldrich, St. Louis, MO), supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France), 2% L-glutamine (Gibco, Carlsbad, CA) and 1% penicillin/streptomycin cocktail (Sigma-Aldrich). The cells were passaged once weekly and provided with fresh medium every second day. Prior to inoculations, the cells were counted using Bürker-chamber and suspended in serum-free RPMI medium.

4.2 Xenograft studies (I-III)

4.2.1 Animals

All xenograft studies were carried out with adult male immunedeficient mice (HSD: Athymic Nude-*Foxn1*^{nu}), purchased from Harlan Laboratories (Indianapolis, IN) and housed in pathogen-free conditions at the Central Animal Laboratory, University of Turku in individually ventilated cages under controlled conditions of light (12h light/12h dark), temperature (21±3ºC) and humidity (55%±15%). The mice were provided with autoclaved tap water *ad libitum* and irradiated RM3 (E) soy-free natural-ingredient feed (Special Diets Services, Essex, UK). All animal experimentations were conducted in accordance with the Finnish Animal Ethics Committee and the institutional animal care policies (license number: 1993/04.10.03/2011), which fully meet the requirements of international guidelines on the care and use of laboratory animals.

4.2.2 Experimental design

In the orthotopic model, one million VCaP cells in 20 µl of RPMI medium were inoculated into the dorsolateral prostate of 5-6 weeks old mice through an abdominal incision. Isoflurane was applied to induce and maintain anesthesia, and for pain relief, mice were injected subcutaneously with buprenorphine (0.05-0.1 mg/kg) before and after the operation, respectively. In the subcutaneous model, two million VCaP cells in 100 µl of RPMI/Matrigel (1:1) suspension were inoculated into the flank of 5-6 weeks old mice. Mice were castrated

removing the testes through a scrotal incision, causing a rapid decline in serum PSA. Buprenorphine (0.05-0.1 mg/kg) was used for analgesia and isoflurane for anesthesia. In the end of the experiment, the mice were euthanized via cervical dislocation. Before the mice were sacrificed, blood samples were collected via cardiac puncture. Tumors were dissected, all dimensions (height, depth, width) of tumors were measured and tumor weights were recorded. The following ellipsoid formula was used for tumor volume calculations: *tumor volume = (π/6) x width x height x length.* Tumor samples were either fixed in 10% formalin and embedded in paraffin or snap-frozen in liquid nitrogen, and then stored in -80°C. Para-aortic lymph nodes were also dissected and fixed in 10% formalin for paraffin embedding.

4.2.3 Castration and antiandrogen treatments

In Study I, mice were distributed into five groups: *Intact 1, Intact 2, GNX, CRPC 1* and *CRPC 2*. Mice were sacrificed individually in different time points based on serum PSA levels of each mouse (Figure 7). *Intact 1* (small tumors, n=7) and *Intact 2* (large tumors, n=7) groups were sacrificed when serum PSA had reached approximately 10 µg/l and 70 µg/l, respectively. The other three groups were castrated, and the mice in *GNX* group (n=8) were sacrificed one day after castration. *CRPC 1* (small tumors, n=7) and *CRPC 2* (large tumors, n=7) groups were sacrificed when the serum PSA was between 10-20 µg/l and 50-90 µg/l, respectively.

In antiandrogen study, the VCaP tumors were let to grow for 4-5 weeks prior to castration. When 60% of the mice had PSA levels higher than 5 µg/l, the mice with tumors were castrated. Before the initiation of antiandrogen treatment, mice were allocated to the treatment arms based on five baseline parameters: serum PSA concentration, the change in the serum PSA concentration, cage placement, body weight and the date of castration. The antiandrogen treatment began when the serum PSA of mice with CR-VCaP (castration-resistant VCaP) tumors had reached the pre-castration levels, mean serum PSA being 15 µg/l. The mice were treated with vehicle (*VEH*, n=15), enzalutamide (*ENZ*, 20 mg/kg/day, n=13) enzalutamide or ARN-509 (*ARN*, 20 mg/kg/day, n=15) for 28 days, administered once a day by gavage.

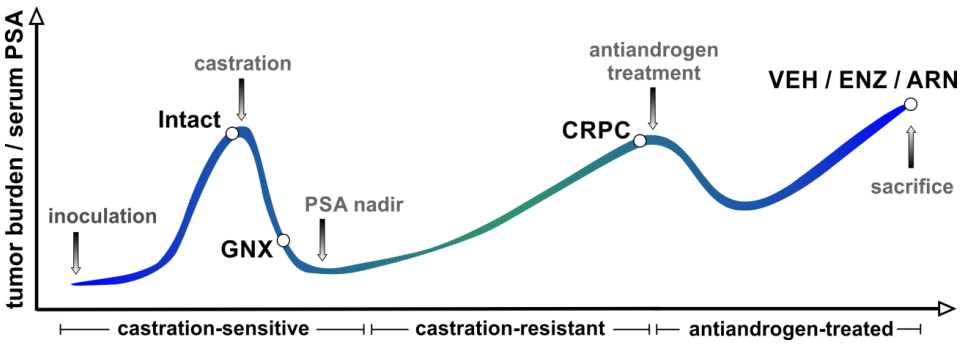

Figure 7. An illustrative timeline of the VCaP xenograft model. Mice in different study groups (*Intact*, *GNX*, *CRPC*, *VEH*, *ENZ* and *ARN*) were sacrificed at different time points (white dots). Serum PSA was used for monitoring tumor growth/burden of VCaP xenografts. The responses to castration and antiandrogen treatments were reflected by decreasing serum PSA levels.

In addition to the orthotopic VCaP xenograft model used in the previously described experiments, the subcutaneous VCaP xenograft model was used to study the effects of a single dose antiandrogen treatment on intratumoral androgen concentrations in the CR-VCaP tumors. The mice with subcutaneous CR-VCaP tumors were treated with a single dose of enzalutamide (n=6, 20 mg/kg) or vehicle (n=6). Tritium labeled dihydrotestosterone ([1,2,4,5,6,7-3H(N)]-^{3}H-DHT, 110 Ci/mmol, PerkinElmer, Waltham, MA) was injected intravenously into the tail vein (~1.6 MBq/mouse) four hours after the single dose of vehicle or enzalutamide was given. ^{3}H-DHT was dissolved in ethanol:saline (20:80) solution. Fifteen minutes after the injection, blood samples were collected, mice were sacrificed and tumor samples were collected. The tumors were homogenized in sterile water using an Ultra Turrax homogenizer (IKA-Werke, Staufen im Breisgau, Germany). Serum samples and tumor homogenates were mixed with Ecoscint A scintillation liquid (National Diagnostics, Atlanta, GA), and the radioactivity of the samples was measured using a Wallac 1410 liquid scintillation counter (PerkinElmer).

4.2.4 Allocation of intervention groups and matching of animals

The matching model was used to allocate and match individual mice into three interventions subgroups (*VEH*, *ENZ*, *ARN*) based on baseline characteristics. Mixed-effects models of the lme4-package (Bates et al., 2015) were utilized to test the differences in the linear PSA growth slopes of VCaP xenografts (Laajala et al., 2012). R statistical software (R Development Core Team, 2008) was used for the analyses.

4.3 Clinical samples (IV)

4.3.1 Patients

Patients diagnosed with localized prostate cancer were operated at Turku University Hospital (TYKS) in the period of 2013-2015. The study protocol was approved by the Ethics Committee of the Hospital District of Southwest Finland and prepared following to the principles in the Declaration of Helsinki. All the patients involved signed a written consent for participating to the study. The study cohort included 48 patients, which were selected among 270 patients. The following inclusion criteria were applied for selecting the patients for the study cohort: age 30-85, a diagnosis of adenocarcinoma in the prostate gland, measurable pre-operative serum PSA levels and post-operative Gleason Score ≥6. The clinicopathological patient information was collected from medical records by an experienced urologist.

4.3.2 Sample collection and validation

Patient samples were collected during robot-assisted laparoscopic prostatectomy (RALP) operations at Turku University Hospital. Blood and urine samples were taken before the operation, aliquoted and stored at -80°C. Tissue samples were collected after the operation from the removed prostate. Prostate dissection was performed at Auria Biobank immediately after the prostate removal. After removing the apex and the base of prostate, three cylinder shaped tissue core samples were taken from the prostate using a MD5000 Tissue Coring Press (Alabama Research & Development, Munford, USA). Each core was cut into three sections, and each section was cut into three tissue samples. The tissue samples were either fixed in formalin for 24 hours or snap-frozen and stored in liquid nitrogen. Next to fresh-frozen tissue samples, one sample of each section was taken to histological validation and evaluated by an experienced uro-pathologist. The amount of cancerous tissue, stromal tissue and benign epithelium as well as the Gleason score and inflammation status of the sample were evaluated. Samples that consist of 50-100 % of confirmed adenocarcinoma were considered as cancerous samples by histological validation. Secondly, tissue samples consisting of various amounts of benign epithelium and stroma but no adenocarcinoma were considered as benign samples. Patients with degraded RNA samples or without corresponding histological validation (cancer and benign) samples were excluded from the study cohort.

4.4 Steroid profiling (I, III, IV)

All steroid analyses were conducted using a LC-MS/MS (liquid chromatography-tandem mass spectrometry or a GC-MS/MS (gas chromatography-tandem mass spectrometry). The methods applied have been validated and described in detail (Keski-Rahkonen et al., 2011; McNamara et al., 2010; Nilsson et al., 2015). The characteristics including lower limit of quantitation (LLOQ) of the LC-MS/MS and GC-MS/MS methodologies are given in Table 1.

Table 1. The characteristics of LC-MS/MS and GC-MS/MS methodologies

	LC-MS/MS (Keski-Rahkonen et al., 2011)	**GC-MS/MS** (Nilsson et al., 2015)	**LC-MS/MS** (McNamara et al., 2010)
Steroid	**LLOQ pg/ml**	**LLOQ pg/ml**	**LLOQ pg/ml**
Pregnenolone	10.4	-	-
Progesterone	10.7	74	-
DHEA	-	50	-
Androstenedione	9.5	12	-
Testosterone	9.5	8	20
DHT	97.6	2.5	100
3α-androstanediol	-	-	400
3β-androstanediol	-	-	400

In Study I, tumor samples from VCaP xenografts were homogenized in sterile Milli-Q water (1:10, weight/volume) using a Tissuelyzer LT homogenizer (Qiagen, Venlo, The Netherlands) with a 5 mm steal bead. For analyzing the serum samples, 150 µl of each serum sample was used for steroid analytics applied in the study (Keski-Rahkonen et al., 2011).

In Study III, all three LC-MS/MS and GC-MS/MS methods (Keski-Rahkonen et al., 2011; McNamara et al., 2010; Nilsson et al., 2015) were utilized to measure a broad panel of steroids. All VCaP xenograft samples were homogenized using a Tissuelyzer LT homogenizer with a 5 mm steal bead.

In Study IV, prostate tissue samples varying 15-50 mg were homogenized in sterile 10 mM KH_2PO_4 buffer (in most cases 1:25, weight/volume) using an Ultra Turrax homogenizer. Next to homogenization, the samples were centrifuged at 800 x g for 10 minutes at 4°C and supernatant samples were collected. Using 100 µl of supernatant, an additional homogenization was performed using a Tissuelyzer LT homogenizer with a 5 mm steal bead at the highest homogenization speed for one minute to improve the sample extraction outcome. Subsequently, homogenates were combined and stored at -80°C. Aliquots of 500

µl of tissue homogenates and 500 µl of serum samples were provided for steroid analytics applied in the study (Nilsson et al., 2015). Homogenate samples were used for one single measurement, whereas serum samples were measured as replicates (250 µl of each). The SHBG levels in serum samples were measured using electrochemiluminescence (ECL) immunoassay method with a Cobas E 602 modular analyzer (Roche Diagnostics, Basel, Switzerland) at Turku University Hospital Laboratory. The amount of non-bound free testosterone in serum was calculated based on measured serum SHBG levels using a method described by Vermeulen et al. (Vermeulen et al., 1999).

4.5 Gene expression profiling

4.5.1 RNA and DNA extraction (I, III, IV)

VCaP tumor samples and prostate tissue samples were homogenized using a Tissuelyzer LT homogenizer and an Ultra Turrax homogenizer, respectively. Total RNA was extracted from VCaP xenograft samples using RNase Mini Kit (Qiagen, Venlo, The Netherlands) following the guidelines provided by the manufacturer and treated with DNase I Amplification Grade (Invitrogen, Carlsbad, CA). For clinical prostate tissue samples collected from patients (Study IV), both total RNA and genomic DNA were extracted with NucleoSpin® RNA kit and NucleoSpin® RNA/DNA buffer set (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. RNA and DNA yield and quality were monitored with a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

4.5.2 RT-qPCR (I, III)

DNase treated RNA samples were reverse transcribed using oligo(dT) primers (Promega, Fitchburg, WI) and M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA). RT-qPCR experiments were carried out using a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) and 2x Dynamo SYBR Green qPCR kit (Thermo Fisher Scientific, Waltham, MA). The expression of genes of interest was normalized to a reference gene, and the relative gene expression was quantified with the Pfaffl-method, taking into account the diverse amplification efficiencies of different RT-qPCR reactions (Pfaffl, 2001). All genes measured with RT-qPCR and their primer sequences are given in Table 2.

Table 2. RT-qPCR primer sequences

Gene	Forward Primer Sequence 5´- 3´	Reverse Primer Sequence 5´- 3´
AKR1C3	GCCAGGTGAGGAACTTTCAC	CAATTTACTCCGGTTGAAATACG
AR-FL	CTTACACGTGGACGACCAGA	GCTGTACATCCGGGACTTGT
AR-V1	CCATCTTGTCGTCTTCGGAAATGTTATGAAGC	CTGTTGTGGATGAGCAGCTGAGAGTCT
AR-V7	CCATCTTGTCGTCTTCGGAAATGTTATGAAGC	TTTGAATGAGGCAAGTCAGCCTTTCT
ASF/SF2	TCTCTGGACTGCCTCCAAGT	GGCTTCTGCTACGACTACGG
CYP17A1	TCTCTGGGCGGCCTCAA	AGGCGATACCCTTACGGTTGT
FKBP5	AAAAGGCCACCTAGCTTTTTGC	CCCCCTGGTGAACCATAATACA
HSD17B6	CTCCAGCATTCTGGGAAGAG	AATATGCTTGGGGGCTTCTT
KLK2	CTGCCCATTGCCTAAAGAAGAA	GGCTTTGATGCTTCAGAAGGCT
KLK3	CCAAGTTCATGCTGTGTGCT	GGTGTCCTTGATCCACTTCC
KLK4	GGCACTGGTCATGGAAAACGA	TCAAGACTGTGCAGGCCCAGC
NOV	ACCGTCAATGTGAGATGCTG	TCTTGAACTGCAGGTGGATG
PMEPA1	TGCCGTTCCATCCTGGTT	AGACAGTGACAAGGCTAGAGAAAGC
RPL19	AGGCACATGGGCATAGGTAA	CCATGAGAATCCGCTTGTTT
ST6GalNAc1	AGGCACAGACCCCAGGAAG	TGAAGCCATAAGCACTCACC
SYTL2	TCTGCCTTGAGAAAACAAACAGTT	GCCAGTGGGTGGCACTAAAA
TMPRSS2-ERG	TAGGCGCGAGCTAAGCAGGAG	GTAGGCACACTCAAACAACGACTGG
UBE2C	AAAAGGCCACCTAGCTTTTTGC	CCCCCTGGTGAACCATAATACA
UGT2B15	CGTTGTGCACATGTACCCTAAAA	CCATGTTCACATTTTCCTTCCTG
UGT2B17	AAGCCTGAAGTGGAATGACCAA	TTTTGTCGCAGGAAAAAGGAAA

4.5.3 Microarray gene expression studies (I)

To study the gene expression changes in VCaP xenograft tumor samples, microarray experiments were performed at the Finnish Microarray and Sequencing Centre using Illumina Human HT-12 v.4 Expression BeadChip (Illumina, San Diego, CA) containing 48,000 probes. The data was quantile normalized and the androgen pathway product analysis by SwitchGear Genomics (Menlo Park, CA) was used to select androgen-regulated genes for further analyses.

4.5.4 RNA sequencing (III, IV)

RNA sequencing (RNA-seq) techniques were applied to VCaP xenografts tumor samples and tissue samples obtained from prostate cancer patients. All the RNA-seq experiments were carried out at the Finnish Microarray and Sequencing Centre, Turku, Finland. Total RNA (300 ng) was used according to Illumina TruSeq® Stranded mRNA Sample Preparation Guide. In Study III, the xenograft samples were sequenced with HiSeq 2500 instrument (Illumina). TruSeq v3 paired-end sequencing chemistry was applied with a 100-base pair read length. In Study IV, the clinical samples were sequenced with HiSeq 3000 instrument

(Illumina) using paired-end sequencing chemistry and 100-base pair read length. The quality check for RNA-seq data was performed using the FastQC tool, the reads were aligned to the human reference genome version hg19 using Tophat software v2.0.10 (III, Kim et al., 2013) or Star software v2.5.0c (IV, Dobin et al., 2013). The number of uniquely mapped reads was counted using HTSeq v0.6.1 with RefSeq annotations (III, Anders et al., 2014) or Subread tool v1.5.0 (IV, Liao et al., 2014). R statistical software (R Development Core Team, 2008) and Bioconductor v2.14 (Gentleman et al., 2004) were used for the downstream analysis of RNA-seq. The trimmed mean of M-values (TMM) approach was used to normalize the library size in the R/Bioconductor package edgeR (Robinson et al., 2010) and RNA-seq data was transformed using the voom approach in the Limma R/Bioconductor package (Ritchie et al., 2015).

Besides global RNA-seq analyses, subset gene lists of AR-interacting genes, steroidogenic enzymes and androgen-regulated genes were selected for further analyses. AR-interacting genes were selected based on the gene list from the androgen receptor gene mutations database (Gottlieb et al., 2012). The gene selection for steroidogenic enzymes was conducted based on Gene Names gene family indexes including the gene families for aldo-keto reductases (AKR), cytochrome P450 enzymes (CYP), short-chain dehydrogenases/reductases (SDR), sulfotransferases (SULT) and UDP-glucuronosyltransferases (UGT). Androgen-regulated genes were selected according to the experiment done by Asangani et al. in which they identified up-regulated genes in VCaP and LNCaP cells upon DHT treatment (Asangani et al., 2014).

4.6 Protein analyses

4.6.1 Sample preparation (I, III)

Samples from xenograft studies were homogenized using an Ultra Turrax homogenizer in ice-cold lysis buffer [150 mM Tris-HCl, 1 mM EDTA, 1 mM SDS, 0.5% sodium deoxycholate, 1% NP-40, 100 µM sodium orthovanadate (Sigma-Aldrich) with a cOmplete Mini protease inhibitor tablet (Roche, Basel, Switzerland)]. Samples were centrifuged for 10,000 g for 20 minutes at 4°C, supernatants were collected, and the total amount of protein concentrations were analyzed using BCA protein assay kit (Pierce, Rockford, IL) and a Wallac 1420 VICTOR2™ microplate reader (PerkinElmer).

4.6.2 Immunoblotting (I, III)

The protein samples were denatured at 95°C, loaded on 4-10% SDS-PAGE gel or 10% precast SDS-PAGE gel (Bio-Rad Laboratories) and separated under reducing conditions. The antibodies used for immunoblotting (IB) are given in Table 3.

In Study I, SDS-PAGE gels were blotted onto Hybond ECL nitrocellulose membrane (GE Healthcare Life Sciences, Little Chalfont, UK) using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories), blocked with 5%-milk-PBS with 0.1% Tween 20 (Sigma-Aldrich) and probed with an anti-AR antibody. After PBS-T washing, the membrane was probed with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (GE Healthcare Life Sciences). The proteins on the membrane were visualized using Western Lightning ECL Pro detection kit (PerkinElmer) and imaged with an ImageQuant LAS 4000 (GE Healthcare Life Sciences). The membrane was stripped with 6M guanidine-HCl solution (0.2% NP-40, 20 mM Tris-HCl) and re-probed with an anti-GAPDH antibody as a reference. ImageJ software v1.47 was used to quantify the intensity of the signal to measure the relative amount of proteins on the membrane.

In Study III, the SDS-PAGE gels were blotted onto iBlot® Transfer Stack membrane using an iBlot® Dry Blotting System (Invitrogen) and probed with anti-AR and anti-AR-V7 primary antibodies. ECL plex goat-anti-Rabbit IgG-Cy5 and anti-mouse IgG-Cy3 were used as secondary antibodies. The proteins on the membrane were visualized using a Typhoon™ laser scanner (GE Healthcare).

Table 3. Antibodies used in studies I, III and IV

Antigen	Manufacturer	dilution	application	study
Androgen receptor (n-20)	Santa Cruz	1:250 / 1:1000	IHC / IB	I,III
Androgen receptor variant V7	Precision Antibody	1:500	IHC	III
GAPDH	HyTest	1:2500	IB	I
ERG	Epitomics	1:250	IHC	IV
ECL plex IgG-Cy5	GE Healthcare	1:2500	IB	III
ECL plex IgG-Cy3	GE Healthcare	1:5000	IB	III
Phospho-histone H3 (pH-H3) ser10	Millipore	1:1500	IHC	I
Vimentin	Dako	1:500	IHC	I

4.6.3 Glucuronidation activity assay (III)

^{3}H-DHT was used as the substrate for each glucuronidation reaction (500,000 cpm/reaction), evaporated under N_2 flow and dissolved in Tris-$MgCl_2$ assay buffer (50 mM Tris, 10 mM $MgCl_2$, 100µg/ml L-α-phosphatidylcholine). For each reaction, 100 µg of total protein from VCaP tumor homogenate samples was used. The volume of each reaction was adjusted with the assay buffer up to 150 µl. Uridine 5'-diphosphoglucuronic acid (UDPGA, 1 mM, Sigma-Aldrich) was used as a cofactor for the reaction and added to the samples. The samples were incubated at 37°C for 4 hours, and immediately after the incubation the reactions were terminated by rapid freezing in liquid nitrogen. To extract the unconjugated ^{3}H-DHT (organic fraction) from the ^{3}H-DHT-glucuronidates (aqueous fraction), diethyl ether (Uvasol®, Merck, Darmstadt, Germany) extraction was performed. The organic fraction samples were evaporated under N_2 flow and dissolved in ethanol. Samples were mixed with 10 ml Ecoscint A scintillation liquid (National Diagnostics), and the radioactivity of the samples were measured using a Wallac 1410 liquid scintillation counter (PerkinElmer).

4.7 Immunohistochemistry (I, III, IV)

All VCaP xenograft tumor samples, para-aortic lymph nodes and prostate samples from patients for immunohistochemistry (IHC) were formalin-fixed and paraffin-embedded. Formalin-fixed samples were sectioned, and the sections were deparaffinized, rehydrated and exposed to the antigen retrieval. The antibodies used for IHC are given in Table 3.

In study I, VCaP tumor samples were stained with primary antibodies against AR, phospho-histone H3 (pH-H3) and vimentin. In addition, para-aortic lymph nodes were stained against the AR. The TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) staining was used to analyze the apoptotic index of the VCaP tumors, using ApopTag Peroxidase *in situ* Apoptosis Detection Kit (Chemicon International, Millipore). In study III, VCaP tumor sections were stained against the AR and AR-V7. After the primary antibody incubation and PBS washing, the endogenous peroxidase activity was blocked with 1% H_2O_2 for 20 minutes.

In study IV, the prostate sections were incubated with a rabbit monoclonal ERG antibody (clone EPR386) for one hour at RT. All the sections were incubated with a secondary anti-rabbit antibody conjugated with polymer-HRP (Dako) at RT. The immunocomplexes were visualized with DAB+ Chromogen solution

(Dako), and the sections were counterstained with Mayer's hematoxylin and mounted.

The pH-H3-positive cells in VCaP tumor sections were quantified by Quva (Tampere, Finland) with their customized image analysis algorithms. Similarly, the apoptotic index of the VCaP tumors was quantified by analyzing the TUNEL-positive cells in VCaP tumor sections. The ERG-stained sections were analyzed by an experienced pathologist. For ERG staining, the vascular endothelial cells were used as a positive control, while the benign glands without the ERG staining served as a negative control. All the stained VCaP tumor sections and clinical prostate sections were digitized using a Pannoramic 250 slide scanner (3DHISTEC, Budapest, Hungary). AR nuclear IHC stainings were quantified using CaseViewer 2.0 + QuantCenter software (3DHISTECH), scoring the nuclei into four categories based on the intensity of the staining.

4.8 PSA measurements (I-III)

The tumor growth of VCaP tumors was monitored measuring the serum levels of PSA secreted by VCaP cells. For PSA measurements, 100 µl of blood was collected by saphenous vein puncture once weekly (Hem et al., 1998). To avoid dehydration, mice were injected with 200 µl of 0.9% NaCl after blood sampling. Blood samples were centrifuged at 3500 x g for 10 minutes at 4°C to collect serum samples for PSA measurements. Serum PSA levels were determined using a time-resolved fluorometric assay applying two antibody sandwich technique, described in detail previously (Lövgren et al., 1996). The amount of PSA in serum samples was determined by measuring the fluorescence signal using a Wallac 1420 VICTOR2™ microplate reader.

4.9 Statistical analyses (I, III, IV)

Basic statistical analyses were performed using SigmaPlot 12.0 statistical software (Systat Software, Chicago, IL) (I) and GraphPad Prism 6.0-7.02 software (GraphPad Software, San Diego, CA) (III-IV). The statistical methods applied in RNA-seq and microarray studies are described separately in sections 4.5.3 and 4.5.4.

Student's t tests and one-way ANOVA with Dunn's post hoc tests were applied to steroid level comparisons (I). One-way ANOVA with Tukey's multiple comparisons was performed to study the differences in the steroid concentrations in VCaP xenografts (I, III). In addition, paired t-test analyses were performed to

compare the androgen concentrations in cancerous and benign tissue samples and serum samples obtained from prostate cancer patients (IV). For a single gene RNA-seq and RT-qPCR analyses, non-parametric Kruskal-Wallis (I, III), Dunn's multiple comparison tests (I, III) and unpaired t-tests (IV) were applied. Holm-Sidak's multiple comparisons test was used to study tumor/serum ratios in vehicle-treated and antiandrogen-treated VCaP xenografts (III). Pearson's correlation coefficient was used to compare tumor volume measurements and terminal PSA values (I), gene expression levels and intratumoral steroid levels (III), serum androgen levels and intraprostatic androgen levels (IV), and gene expression levels measured by RNA-seq (IV).

5 RESULTS

5.1 Characterization of orthotopic VCaP xenograft model

5.1.1 Castration-resistant growth of the VCaP xenografts

The growth of orthotopic VCaP tumors was monitored by measuring serum PSA in mice once a week throughout the experimental periods. Because there is no endogenous PSA secretion in mouse, serum PSA was considered to be a reliable tool for monitoring the tumor growth in the orthotopic VCaP xenograft model. In line with this reasoning, the terminal serum PSA concentrations correlated significantly with tumor volume in VCaP tumors collected from intact and castrated mice (I: Fig. 1B). Two weeks after the inoculation of VCaP cells, the PSA levels were measured for the first time. The detectable levels (>0.5 µg/l) were observed in approximately 70% of mice. The mice that were castrated/ orchidectomized (ORX) 2-5 weeks after the inoculation showed an immediate decline in serum PSA, measured one day after castration (Table 4). The PSA nadir was reached one week after castration, and the PSA levels started to increase in two weeks after castration, reaching the pre-castration levels in 5-6 weeks on average (Table 4). No difference was found in the concentrations of intratumoral PSA (ng/µg of total protein) between the intact and castrated mice measured 3-14 weeks after castration (I: Fig. 1C).

5.1.2 Antiandrogen treatments

The effects of antiandrogens (enzalutamide and ARN-509) on the growth of the orthotopic CR-VCaP tumors were monitored using serum PSA measurements. Despite the clear response after one week of antiandrogen treatment, no differences were found in the tumor volume or terminal serum PSA levels between the vehicle-treated and antiandrogen-treated CR-VCaP tumors (I: Supp. Fig. 2B). However, both enzalutamide and ARN-509 showed a significant longitudinal response on the growth of the CR-VCaP tumors as the serum PSA levels of antiandrogen-treated mice decreased over the 4-week treatment period (Table 4; I: Fig. 5; III: Fig. 1A). The efficiency of treatments was evaluated by comparing differences in longitudinal PSA levels between the mice that were matched before the initiation of the 4-week treatment period. Almost all matched pairs showed a decrease in the PSA growth slope in antiandrogen-treated mice compared with vehicle-treated mice (I: Fig. 5).

Table 4. Serum PSA levels in VCaP xenografts

		Mean serum PSA (µg/l) ± SD					**Time (days)**	
Group	n	Pre-ORX (Intact)	ORX (1 day)	ORX (1 week)	CRPC	Antiandrogen treatment	Pre-ORX	Post-ORX
Intact 1	7	***12.0 ± 3.60***	-	-	-	-	21-63	-
Intact 2	7	***78.2 ± 29.97***	-	-	-	-	21-98	-
GNX	8	15.7± 10.58	***7.6 ± 4.18***	-	-	-	21-42	1
CRPC 1	7	12.7 ± 4.59	-	1.7 ± 0.84	***13.6 ± 3.62***	-	21-42	21-84
CRPC 2	7	16.4 ± 4.45	-	6.0 ± 6.07	***73.5 ± 11.37***	-	21-42	70-98
VEH	15	20.9 ± 6.86	-	2.0 ± 1.26	15.2 ± 7.35	***53.39 ± 29.49***	29-38	62-70
ENZ	13	20.1 ± 6.05	-	1.8 ± 1.28	14.5 ± 6.41	***38.44 ± 23.96***	29-38	62-70
ARN	15	24.5 ± 6.59	-	2.0 ± 0.84	14.4 ± 6.52	***22.88 ± 13.52***	29-38	62-70

5.1.3 Molecular characteristics of the VCaP xenografts

In study I, cell proliferation and apoptosis were studied in the VCaP tumors collected from intact and castrated mice using phospho-histone H3 staining and TUNEL staining, respectively. The characteristics of small VCaP tumors (*Intact 1*, *CRPC 1*) and large VCaP tumors (*Intact 2*, *CRPC 2*) were analyzed. Compared with small and large CRPC tumors and large tumors from intact mice, the proliferation index was higher in the small VCaP tumors collected from intact mice (I: Supp. Fig. 1). No differences in apoptosis were observed between the VCaP tumors collected from intact and CRPC tumors (I: Supp. Fig. 1). All VCaP tumors were negative for vimentin which is an epithelial-mesenchymal transition (EMT) marker. In addition, possible metastasis sites of VCaP xenografts were studied. However, no macroscopic or microscopic metastases in para-aortic lymph nodes were found in any of the VCaP xenografts.

5.1.4 Optimization of experimental design and statistical analyses

A novel approach to optimize experimental design and statistical analyses for preclinical animal studies was developed using the VCaP xenograft model. Using this matching model, the mice bearing CR-VCaP tumors were allocated to optimal intervention groups based on two baseline characteristics: body weight

and serum PSA. Subsequently, the optimal matches of animals were randomized into three interventions groups (II: Fig. 2). As a result, the method allowed for unbiased allocations of animals into treatment arms as matching and randomization could be performed independently of one another. A comparison of the unmatched and matched analyses using the paired longitudinal differences in serum PSA levels demonstrated that the matching approach enhanced the statistical inference of antiandrogen treatment responses (II: Fig. 3). Interestingly, the antiandrogenic effect of the enzalutamide treatment was statistically significant when matching was utilized with mixed-effects modeling, while the ARN-509 treatment effect was significant in both the unmatched and matched models (II: Fig. 3). Furthermore, the matching method successfully normalized the confounding baseline variability among the VCaP xenografts. Additionally, the matching of animals improved the statistical power with which statistical analyses could detect significant treatment effects in the castration-resistant VCaP xenografts using a smaller sample size (II: Fig. 4A).

5.2 Steroid levels in VCaP xenografts and clinical samples

The concentrations of pregnenolone, progesterone, A-dione, T, DHT, 3α-diol and 3β-androstanediol (3β-diol) in VCaP xenografts samples were analyzed utilizing three different LC-MS/MS and GC-MS/MS methods.

5.2.1 Intratumoral and serum levels of steroids in the VCaP xenografts

Both serum and intratumoral concentrations of A-dione, T and DHT were measured in intact and castrated mice using a LC-MS/MS. The levels of A-dione and T were detectable in the sera of intact mice (*Intact 1, Intact 2*), whereas the serum DHT levels in all study groups were below the quantification limit of the LC-MS/MS method applied in Study I (I: Fig. 2). As expected, because of castration, A-dione and T levels were dramatically reduced, being undetectable in the sera of mice sacrificed one day after castration (*GNX*) and in the mice with CR-VCaP tumors (*CRPC 1, CRPC 2*). Interestingly, intratumoral concentrations of A-dione, T and DHT were measurable in the VCaP tumors grown in both intact and castrated mice. The intratumoral levels of androgens, T and DHT were transiently reduced one day after castration but recovered back to the pre-castration levels in the CR-VCaP tumors (I: Fig. 2). As a result, no differences in intratumoral T and DHT levels were observed in the CR-VCaP compared with the tumors grown in intact mice, indicating an up-regulation of intratumoral androgen biosynthesis in the CR-VCaP tumors (I: Fig. 2).

5.2.2 The effects of antiandrogens on intratumoral steroid levels

Remarkably, compared with the levels measured in vehicle-treated CR-VCaP tumors (*VEH*) or VCaP tumors grown in intact mice (*Intact*), the enzalutamide (*ENZ*) and ARN-509 (*ARN*) treatments significantly reduced the intratumoral concentrations of T and DHT in the CR-VCaP tumors (III: Fig. 2A; Figure 8). In addition, A-dione concentrations were detected at measureable levels in the CR-VCaP tumors but remained unaffected after the antiandrogen treatment (III: Fig. 2A). Furthermore, the concentrations of 3α-diol and 3β-diol, the primary downstream metabolites of androgens, were analyzed in vehicle-treated and antiandrogen-treated CR-VCaP tumors. However, these androgen metabolites were not detected in the samples because of the assay's sensitivity limit, indicating that the reduction of intratumoral T and DHT in the antiandrogen-treated CR-VCaP tumors was not compensated for by a similar increase in 3α-diol and 3β-diol concentrations. Moreover, intratumoral pregnenolone levels remained unchanged in all treated CR-VCaP tumors, whereas intratumoral progesterone levels were significantly decreased in the enzalutamide-treated CR-VCaP tumors compared with the levels measured in vehicle-treated tumors (III: Fig. 2A). The serum steroid concentrations (pregnenolone, progesterone, A-dione, T and DHT) of mice with vehicle-treated or antiandrogen-treated CR-VCaP tumors were measured, showing no significant differences between the mice (III: Fig. 2A).

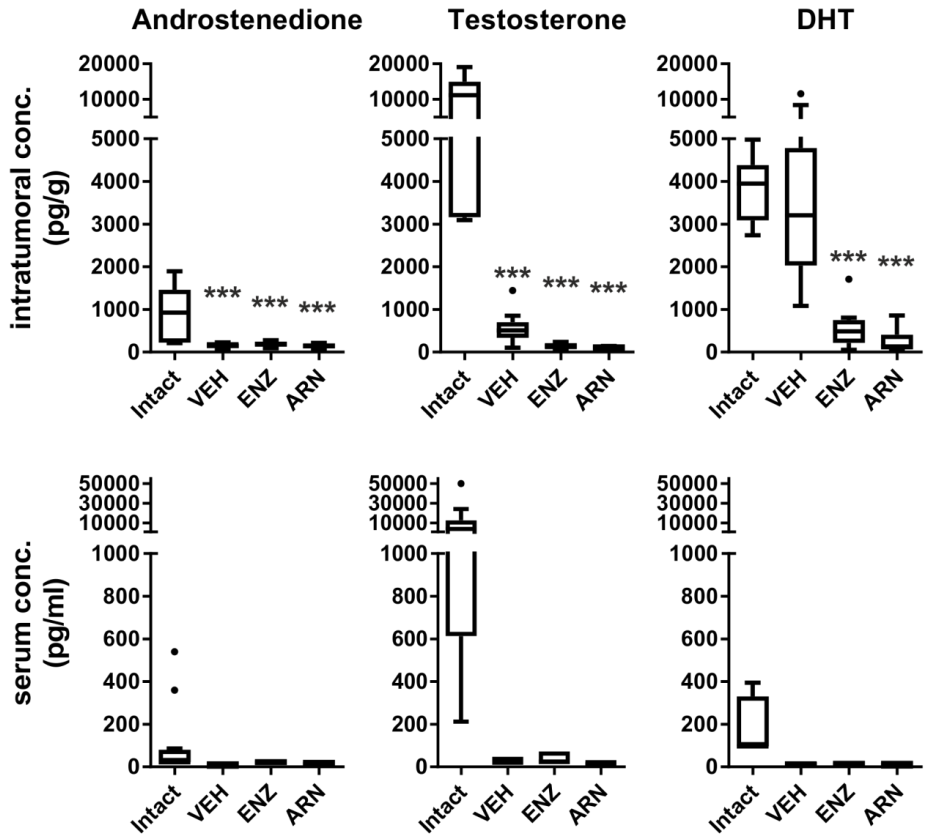

Figure 8. An overview of intratumoral and serum androgen concentrations in VCaP xenografts. Intratumoral (pg/g) and serum levels (pg/ml) of androstenedione, testosterone and DHT were measured using a GC-MS/MS. Samples of *Intact* group and *VEH*, *ENZ* and *ARN* groups were collected from two different animal experiments, however, tumor and serum samples for steroid analyses were prepared simultaneously and measured at the same time. In this figure, the androgen levels of CR-VCaP xenografts (*VEH*, *ENZ* and *ARN*) are compared to/ *Intact* group. Adjusted p-values: *** $p<0.001$

5.2.3 Intraprostatic androgen levels in clinical specimens

The concentrations of DHEA, A-dione, T and DHT were measured using a GC-MS/MS in prostate tissue and serum samples obtained from 48 patients diagnosed with primary prostate cancer. Among all patients included in the study cohort, 38 patients did not receive any hormonal therapies, 9 patients were treated with 5α-reductase inhibitors (either finasteride or dutasteride) and one patient was treated with GnRH antagonist (degarelix) for pre-operative ADT (IV: Table 1). The intraprostatic androgens levels of histologically confirmed cancerous and benign prostate samples were analyzed for each patient. The

median intraprostatic levels of DHT in patients without any hormonal therapies were significantly higher in the cancer samples than in the benign samples (IV: Table 2). However, no differences in DHEA, A-dione or T concentrations were observed between the cancer and benign tissue samples (IV: Table 2). The results demonstrated the high intraprostatic variance of different androgens between the patients (IV: Fig. 1B, 1C). Moreover, as expected, the hormonal therapies significantly altered the intraprostatic androgens levels in patients who were treated with 5α-reductase inhibitors or GnRH antagonist (IV: Table 2). The intraprostatic levels of DHT increased, whereas the intratumoral T levels decreased in the patients treated with 5α-reductase inhibitors (IV: Table 2).

5.2.4 TMPRSS2-ERG and intratumoral androgen levels

Intraprostatic androgen levels in cancerous and benign samples were normalized to each other, and the cancer/benign ratio was used to analyze the differences between the patients. Using unsupervised hierarchical clustering, the patients were clustered into subgroups, revealing a distinct difference between TMPRSS2-ERG fusion positive (ERG+) and TMPRSS2-ERG fusion negative (ERG-) tumors (IV: Fig. 3A). The intratumoral and serum levels of T were decreased in patients with ERG+ tumors, whereas the levels observed in benign samples remained unaffected (IV: Fig. 3B). Furthermore, the DHT/T ratios in ERG+ cancerous samples were prominently increased compared with those in ERG- samples, while no differences in the DHT/T ratios of benign and serum samples were observed (IV: Fig. 3C).

5.3 Gene expression levels of steroidogenic enzymes

The expression levels of enzymes synthesizing androgens and other steroids were studied in the VCaP xenograft model and clinical samples obtained from prostate cancer patients. Commonly referred to as steroidogenic enzymes, most of these enzymes belong to the gene families of cytochrome P450 (CYP) enzymes, short-chain dehydrogenases/reductases (SDR), aldo-keto reductases (AKR), sulfotransferases (SULT) and UDP-glucuronosyltransferases (UGT). The gene expression levels of these enzymes in each study (I, III, IV) were evaluated using RT-qPCR, RNA-seq or/and microarray techniques.

5.3.1 Steroidogenic enzymes in the VCaP xenografts

The expression of several critical steroidogenic enzymes was confirmed in the VCaP xenograft tumors using RT-qPCR. The expression of steroidogenic enzymes CYP17A1, AKR1C3 and HSD17B6 was up-regulated in the VCaP tumors immediately after castration but also in the castration-resistant stage (I: Fig. 3A). In contrast, the antiandrogens decreased the expression of HSD17B6 in the CR-VCaP tumors (III: Fig. 3B). In addition to these analyses, both microarrays and RNA sequencing studies were performed to investigate the gene expression in the VCaP tumors. The mRNA expression levels of AKR-, CYP-, SDR- SULT- and UGT-enzymes were analyzed in vehicle-treated and antiandrogen-treated CR-VCaP tumors using RNA-seq. Out of 258 genes coding for these enzymes, 29 genes were found to be differentially expressed in either enzalutamide-treated or ARN-509-treated VCaP tumors compared with vehicle-treated VCaP tumors (III: Fig. 3A). No significant gene expression changes were observed in classical steroidogenic enzymes such as CYP11A1, CYP17A1, HSD3B1, HSD3B2 and SRD5A1, however, the mRNA expression levels of CYP1B1, CYP4F12 and TBXAS1 were significantly decreased by enzalutamide and ARN-509 treatments (III: Fig. 3B). The gene expression of UGT2B4 and UGT2B11, enzymes forming polar steroid-glucuronides, was also reduced by both antiandrogen treatments (III: Fig. 3B). Interestingly, the expression of UGT2B15 and UGT2B17 was prominently up-regulated after castration (Figure 9, unpublished data). Nevertheless, the gene expression levels of UGT2B15 and UGT2B17 decreased back to the pre-castration levels in the non-treated and antiandrogen-treated CR-VCaP tumors.

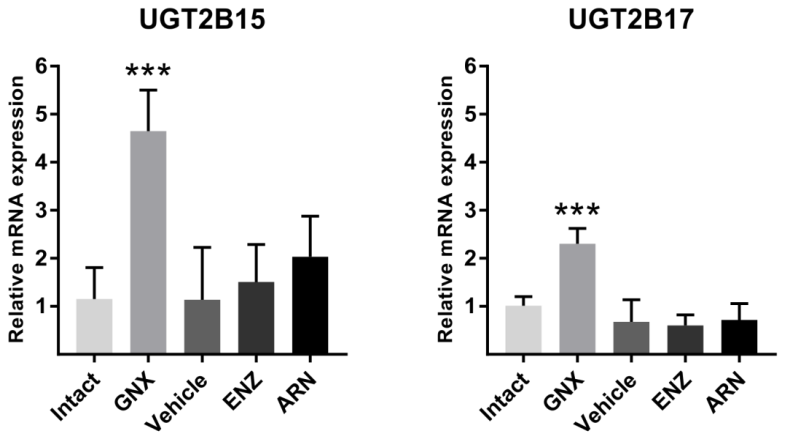

Figure 9. The relative expression of UGT2B15 and UGT2B17 in VCaP tumors. The mRNA levels measured by RT-qPCR were compared to *Intact* group. *** $p<0.001$

5.3.2 Steroidogenic enzymes in clinical prostate specimens

Several steroidogenic enzymes were found to be differently expressed between the ERG+ and ERG- tumors using RNA-seq analyses. The gene expression of DHRS9, HSD11B1 and HSD17B6 was significantly decreased in ERG+ tumors compared with that in ERG- tumors (IV: Fig. 5A). Furthermore, the expression levels of SRD5A3 were significantly increased in the ERG+ tumors compared with those in ERG- tumors (IV: Fig. 5A). In contrast, none of these changes were observed in the benign samples obtained from the same patients (IV: Fig. 5A).

5.4 The AR and AR splice variants in the VCaP xenografts

The expression of AR-FL was up-regulated in the VCaP tumors immediately after castration, and AR-FL expression was further induced up to 20-fold in the CR-VCaP tumors (I: Fig. 4A). Similarly, castration induced an up-regulation of AR splice variants AR-V7 and AR-V1 compared with the levels measured in the VCaP tumors grown in intact mice (I: Fig. 4A). Furthermore, AR-FL, AR-V1 and AR-V7 mRNA levels were further increased after the enzalutamide or ARN-509 treatments (III: Fig. 1B). AR-FL mRNA was increased approximately 2-fold, whereas the AR-V1 and AR-V7 splice variants were up-regulated 2-3-fold in the antiandrogen-treated CR-VCaP tumors compared with the levels measured in the vehicle-treated CR-VCaP tumors (III: Fig. 1B). The up-regulation of AR protein levels was also demonstrated in the CR-VCaP tumors using immunoblotting and IHC analyses (I: Fig. 4B, 4C). Antiandrogens further increased both AR-FL and AR-V7 protein levels based on immunoblotting analyses (III: Fig. 1C). Furthermore, IHC studies demonstrated that the AR is localized in the nucleus in the presence of the antiandrogens (III: Fig. 1D).

The effects of antiandrogens on the mRNA expression of AR-interacting proteins were studied. Global RNA-seq analysis revealed that 291 genes were markedly down-regulated or up-regulated by one or both antiandrogens (III: Supp. Fig. 1). Accordingly, 67 genes of those genes encoded different AR-interacting proteins, indicating remarkable rearrangements in the transcriptional machinery mediating AR signaling (III: Fig. 1F). Nevertheless, the gene expression levels of several AR splicing factors, including SRSF1 (ASF/SF-2) and AR-V target gene UBE2C, were not altered by antiandrogens (III: Fig. 1E).

5.5 Androgen-regulated gene expression

5.5.1 AR target gene regulation in the VCaP xenografts

The gene expression of classical androgen-regulated genes KLK2, KLK3 and KLK4 was altered in the VCaP xenografts based on RT-qPCR analyses (I: Fig. 3B). KLK2 and KLK3 levels were decreased after castration, however, their mRNA expression levels were increased to the pre-castration levels in the CR-VCaP tumors (I: Fig. 3B). In addition, the expression of the TMPRSS2-ERG fusion gene was regulated in a similar manner in line with KLK2 and KLK3 expression (I: Fig. 3B) Using microarray analysis, 49 androgen-regulated genes were found to be differentially expressed when comparing the *Intact*, *GNX* and *CRPC* tumors. The unsupervised clustering based on microarray data demonstrated that both small and large VCaP tumors collected from intact mice and the CR-VCaP tumors clustered together (I: Fig. 3C). Accordingly, no clear differences in the androgen-regulated gene expression levels were observed between the small (*Intact 1, CRPC 1*) and large tumors (*Intact 2, CRPC 2*). The most drastic gene expression alterations were found in the *GNX* group, which was clearly different from the VCaP tumors collected from the *Intact* and *CRPC* groups (I: Fig. 3C).

5.5.2 The effects of antiandrogens on AR target gene expression

The effects of antiandrogens were analyzed in the CR-VCaP tumors by RNA-seq. The hierarchical clustering revealed that the vehicle-treated and antiandrogen-treated tumors clustered separately, indicating differences in androgen target gene expression (III: Supp. Fig. 1). The RT-qPCR analyses in conjunction with RNA-seq data verified the altered androgen-regulated gene expression in the antiandrogen-treated CR-VCaP tumors (III: Fig. 4). However, well-known androgen target genes such as KLK3 or TMPRSS2-ERG fusion were not altered, whereas the expression levels of many other well-characterized androgen-regulated genes, including FKBP5, PMEPA1 and KLK2, were down-regulated by antiandrogens (III: Fig. 4A, 4C). In addition to these androgen-regulated genes, the expression of a few AR splice variant-specific target genes, such as CAMK2N1, GUCY1A3 and IGFBP3, were altered in the antiandrogen-treated VCaP tumors (III: Fig. 4D).

The global RNA-seq analyses demonstrated that the most up-regulated and down-regulated genes by antiandrogens were androgen-regulated genes (III: Supp. Tables 1-4). Neuroblastoma-overexpressed (NOV) was the most up-

regulated gene, whereas the most down-regulated gene, ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 1 (ST6GalNAc1), was down-regulated almost 10-fold (III: Fig. 4B). Interestingly, the high expression levels of NOV correlated significantly with AR expression levels (III: Fig. 4B).

5.5.3 AR target gene expression in clinical specimens

The AR target gene expression was profiled in the clinical prostate samples (cancerous and benign) using RNA-seq. The altered androgen-regulated genes were visualized by unsupervised hierarchical clustering, demonstrating a clear separation between the ERG+ and ERG- tumors (IV: Fig. 5B). The expression of 31 androgen-regulated genes was altered, including such genes as NKX3-1, SPOCK1 and STEAP4, which were overexpressed in ERG+ tumors compared with the levels measured in ERG- tumors (IV: Fig. 5C).

5.6 TMPRSS2-ERG fusion expression

The TMPRSS2-ERG fusion levels in the VCaP tumors followed the same expression patterns observed for classical androgen-regulated genes such as KLK3. The TMPRSS2-ERG transcript levels were decreased after castration but eventually increased to the levels comparable to those observed before castration (I: Fig. 3B). Antiandrogen treatments did not have any effects on the TMPRSS2-ERG transcript levels in the CR-VCaP tumors (III: Fig. 4A).

The TMPRSS2-ERG fusion status of ERG+ and ERG- clinical tumor samples was determined by measuring the mRNA expression of ERG using RNA-seq (IV: Fig. 2A). To confirm the RNA-seq findings, ERG was stained in 5 tumors using IHC. ERG expression was confirmed by IHC as high ERG expression, which translated to positive ERG immunostaining, whereas the samples with no ERG expression were negative for ERG immunostaining (IV: Fig. 2C). The high ERG expression levels were associated with high CRISP3 expression in clinical tumor samples (IV: Fig. 2B).

6 DISCUSSION

6.1 Characterization of the VCaP xenograft model

To study intratumoral androgen biosynthesis, AR action and novel CRPC therapies targeting androgen signaling, an orthotopic VCaP prostate cancer xenograft was developed as a part of the thesis. The VCaP cell line was chosen because it possesses several features that make it superior to other cell lines for the intended purpose. Due to AR amplification, androgen-sensitive VCaP cells express high levels of wild-type AR, and they secrete PSA, which are both typical features of prostate cancer (Korenchuk et al., 2001). Unlike most of the prostate cancer cell lines available, VCaP cells harbor TMPRSS2-ERG translocation found in approximately 50% of all prostate cancer tumors (Tomlins et al., 2005). The VCaP cell line expresses the steroidogenic enzymes (Cai et al., 2011), and is sensitive to androgen-regulated modulations (Cheng et al., 2010), making it an useful prostate cancer model for studying intratumoral androgen biosynthesis, which is one of the key features in clinical CRPC. Nevertheless, the VCaP cell line represents a singular CRPC subtype, thus, PDX models such as the LuCaP series are essential in displaying the molecular heterogeneity of CRPC in preclinical studies (Nguyen et al., 2017).

Androgen modulation by castration and antiandrogen treatment caused dramatic changes in the serum PSA levels of VCaP xenografts. Serum PSA measurements were observed to be a useful tool for monitoring the growth of VCaP tumors. The terminal serum PSA concentrations correlated significantly with the VCaP tumor sizes at the time of sacrifice, thus, the data strongly suggest that decreases in serum PSA are related to decreases in tumor size. Given the limitation that orthotopic tumors could not be measured by palpation during the study, this result cannot be fully confirmed. However, it is possible that antiandrogens modulate serum PSA levels more dynamically but do not dramatically affect the tumor volume, as demonstrated in another prostate cancer xenograft model with suramin treatment (Thalmann et al., 1996). Furthermore, the similar proliferation and apoptosis rates observed in non-castrated and castration-resistant VCaP xenografts suggest that CR-VCaP tumors do not exhibit a more aggressively growing phenotype compared with VCaP tumors collected from intact mice.

6.1.1 Orthotopic vs subcutaneous VCaP models

In contrast to most studies that have utilized subcutaneous VCaP xenograft models (Loberg et al., 2006; Cai et al., 2009; Yu et al., 2014), we established an

orthotopic VCaP xenograft model in which the VCaP cells are inoculated into the dorsolateral lobe of the mouse prostate. Our results are comparable to those obtained in a study conducted with a subcutaneous VCaP CRPC xenograft model, in which a 5-week treatment of enzalutamide (10 mg/kg) or ARN-509 (10 mg/kg) significantly reduced VCaP tumor growth (Asangani et al., 2016). In addition, one of the reasons why we chose to establish an orthotopic model was the ability study the formation of local metastases and effects of novel CRPC therapies. VCaP cells are originally derived from a vertebral metastasis (Korenchuk et al., 2001), however, we observed no visible metastases or micrometastases in proximate para-aortic lymph nodes in any VCaP xenografts. To our knowledge, no signs of metastases in VCaP xenograft models have been reported, however, Asangani et al. detected genomic DNA of VCaP cells in femur and liver samples obtained from antiandrogen-treated VCaP xenografts, indicating micrometastases in these tissues (Asangani et al., 2014).

Taken together, it is not trivial to determine whether the orthotopic VCaP model is better suited for studying intratumoral androgen biosynthesis than similar subcutaneous models. Despite the technical limitations not shared by subcutaneous models, the orthotopic VCaP model embodies the genuine prostate microenvironment. The cross-talk between epithelial cells and stromal cells has been demonstrated to be crucial for the development and progression of prostate cancer (Niu & Xia, 2009), thus, the orthotopic model more accurately reflects human prostate cancer because of the presence of mouse stroma.

6.1.2 Matched analysis of orthotopic VCaP xenografts

A matching algorithm was developed to optimize the experimental and statistical design of preclinical animal studies. In general, the matching of mice in the antiandrogen treatment study with VCaP xenografts improved the study outcome by balancing the treatment arms based on baseline variables and boosting the power of statistical analyses. The matching of animals was useful for optimizing the study design before the initiation of antiandrogen treatments, but it also improved the post-intervention statistical analyses, distinguishing the pure antiandrogen intervention effects from background noise. The results demonstrated that controlling the individual variation of mice and careful experimental and statistical design in preclinical animal models are essential for improving the reproducibility of animal experimentation and translational efforts towards clinical trials, as also acknowledged by others (Kilkenny et al., 2013; Muhlhausler et al., 2013). In addition to conventional xenograft models, the matching approach can be applied to PDX models, in which it is even more

critical to account for heterogeneous tissue material and individual differences between patients (Day et al., 2015).

6.2 Intratumoral androgen biosynthesis in VCaP tumors

In the VCaP xenograft model, castration dramatically decreased both intratumoral and serum levels of androgens, namely, A-dione, T and DHT. However, intratumoral androgen levels decreased only transiently after castration as significant concentrations of T and DHT were measureable in the CR-VCaP tumors despite the low or undetectable levels of androgens in the sera of mice. This observation indicates an induction of intratumoral androgen biosynthesis after castration, independently of gonadal androgen production, suggesting that the intratumoral levels of T and DHT are most likely synthesized either from adrenal precursors or *de novo* from cholesterol in VCaP tumors (Cai & Balk, 2011; Penning, 2014).

Studies focused on intratumoral androgen biosynthesis in prostate cancer have identified different steroidogenic routes, namely, canonical, alternative and backdoor pathways, to synthesize androgens in CRPC tumors (Chang et al., 2011; Mohler et al., 2011b; Fiandalo et al., 2014). These three pathways require several classical enzymes of steroid biosynthesis, such as CYP11A1, HSD3B2 and CYP17A1. The gene expression profiling of CR-VCaP tumors using RNA-seq revealed that most of the upstream enzymes, including CYP11A1, HSD3B1 and HSD3B2, were not substantially expressed in the CR-VCaP tumors. The lack of enzymes required for *de novo* androgen biosynthesis, together with LC-MS/MS and GC-MS/MS data confirming detectable levels of androgen precursors in the sera of castrated mice, suggests that instead of *de novo* androgen biosynthesis, intratumoral androgens are synthesized from the adrenal androgen precursors in CR-VCaP tumors. These observations are in line with studies conducted by Kumagai et al., in which they demostrated that VCaP cells convert adrenal androgen precursors to active androgens *in vitro* but are not capable of *de novo* androgen biosynthesis (Kumagai et al., 2013a). Further studies are needed to address the role of adrenal androgens in the orthotopic VCaP xenograft model.

Intratumoral DHT concentrations were particularly high in the CR-VCaP tumors, whereas intratumoral T levels varied notably in the non-treated CR-VCAP tumors. The results might suggest that DHT biosynthesis was redirected towards the alternative and/or backdoor pathway. Whether A-dione is converted to T or 5α-dione depends on the relative expression and activity of 17β-HSD and 5α-reductase enzymes. In several prostate cancer cell lines, including VCaP cells,

the conversion from A-dione to DHT via 5α-dione has been demonstrated to be the predominantly active pathway, suggesting that the alternative pathway via 5α-dione drives the progression of CRPC (Chang et al., 2011). Furthermore, Chang et al. observed that the conversion from A-dione to 5α-dione is preferably mediated by SRD5A1, not SRD5A2, which is the major 5α-reductase present in the normal prostate (Luu-The et al., 2008). SRD5A1 was also expressed exclusively in the CR-VCaP tumors, as no SRD5A2 expression was found in our analyses. Therefore, it is feasible that CR-VCaP tumors could bypass testosterone as an intermediate and synthesize DHT via an alternative pathway when the source of circulating T produced in gonads vanishes because of castration.

6.2.1 AKR1C3 and HSD17B6 - drug targets for novel CRPC therapies

The expression of steroidogenic genes AKR1C3 and HSD17B6 was up-regulated in the CR-VCaP tumors, indicating an induction of intratumoral androgen biosynthesis. The activity of AKR1C3 has been associated with the progression of CRPC, thus, AKR1C3 has become a potential drug target for intratumoral androgen biosynthesis (Stanbrough et al., 2006; Pfeiffer et al., 2011; Adeniji et al., 2013). AKR1C3 acts as a HSD17B enzyme, similarly to HSD17B3. Both enzymes catalyze reactions that synthesize T and DHT from their inactive precursors A-dione and 5α-dione, respectively. Whereas the expression of AKR1C3 is abundant in CR-VCaP tumors, HSD17B3 is absent in VCaP cells. Thus, AKR1C3 likely regulates the biosynthesis of active androgens either via classical or alternative pathways, both of which lead to the accumulation of intratumoral DHT in CR-VCaP tumors. Similarly to AKR1C3, HSD17B6 has been suggested to play an important role in CRPC by converting 3α-diol to DHT via the backdoor pathway (Mohler et al., 2011a; Mohler et al., 2011b). Intratumoral androgen biosynthesis via the backdoor pathway appears plausible in CR-VCaP tumors because all the necessary enzymes are present. However, a broader platform of steroid metabolites, including backdoor pathway metabolites, should be measured from CR-VCaP tumors to confirm the role of the backdoor pathway. Nevertheless, the up-regulation of AKR1C3 and HSD17B6, together with significant intratumoral T and DHT concentrations, support the critical role of these enzymes in intratumoral androgen biosynthesis in CR-VCaP tumors. Thus, the VCaP model could be a useful preclinical model for studying the effects and efficiency of novel CRPC therapies inhibiting steroidogenic enzymes, particularly the activity of AKR1C3 or HSD17B6.

6.2.2 Androgen glucuronidation

The inactivation of androgens by glucuronidation is one of the mechanisms that regulate ligand availability. DHT and its metabolites are inactivated by the glucuronidation reaction, particularly by UGT2B15 and UGT2B17 enzymes (Bélanger et al., 2003). The AR directly interacts with the UGT2B15 and UGT2B17 genes by binding to their promoter regions (Bao et al., 2008). In addition, the UGT2B15 gene has been identified as a negatively regulated AR target gene in prostate cancer cells (Pâquet et al., 2012). Based on these recent findings, androgens are capable of regulating the glucuronidation processes in CRPC cells via the AR (Gauthier-Landry et al., 2015). We observed a prominent overexpression of the UGT2B15 and UGT2B17 enzymes in the VCaP tumors one day after the mice were castrated. The up-regulation of these enzymes involved in androgen inactivation is, in fact, paradoxical as the activation of intratumoral androgen biosynthesis is associated with the progression of CRPC. These results indicate that decreased intratumoral androgen concentrations after castration are reflected by increased gene expression levels of UGT2B15 and UGT2B17. Whether this up-regulation results in an increase in androgen glucuronidation in VCaP tumors remains unclear.

6.3 Androgen levels in antiandrogen-treated VCaP tumors

Different mechanisms that could explain the reduced androgen levels observed in the antiandrogen-treated CR-VCaP tumors were explored. Enzalutamide and ARN-509 treatments altered the expression of several steroidogenic enzymes, including AKR1C2 and HSD17B6. AKR1C2 converts DHT to a weak androgen metabolite 3α-diol (Bauman et al., 2006b), whereas HSD17B6 can convert 3α-diol to DHT (Ishizaki et al., 2013). Accordingly, the reduced T and DHT concentrations in the antiandrogen-treated CR-VCaP tumors could have been a result of enhanced conversion of DHT to 3α-diol. However, the present data indicate that the decline in DHT concentration was not associated with a corresponding increase in 3α-diol concentrations. It should be noted that the relatively high LLOQ of the assay precluded the detection of minor changes in 3α-diol concentrations.

The reduction of active androgens in the CR-VCaP tumors by antiandrogens raised the question concerning whether the glucuronidation of androgens is increased. However, neither the RNA-seq analyses nor the enzymatic activity experiments provided evidence for increased glucuronidation activity in the CR-VCaP tumors. Instead, some of the UDP-glucuronosyltransferases, such as

UGT2B4 and UGT2B11, were down-regulated in the antiandrogen-treated CR-VCaP tumors. There is no evidence demonstrating that these enzymes conjugate T and DHT, but UGT2B4 has been shown to conjugate several other steroids, such as catechol-estrogens, 3α-diol and androsterone (Lévesque et al., 1999).

Despite the efforts undertaken, no clear explanation was obtained for the reduced testosterone and DHT levels in the antiandrogen-treated CR-VCaP tumors. While the VCaP model exhibits the hallmarks of CRPC, it is not known whether the effects of antiandrogens on intratumoral androgen levels translate into clinical CRPC as similar findings in patients are scarce. However, a recent open-label neoadjuvant study demonstrated that patients who were treated with GnRH agonist or GnRH antagonist (degarelix) combined with antiandrogen (bicalutamide) prior to radical prostatectomy were associated with lower intratumoral DHT levels compared with patients who were treated with GnRH antagonist monotherapy (Sayyid et al., 2016). In addition, no differences in intratumoral T levels were observed between the treatment groups with or without antiandrogen, but it is possible that differences were not detected because of a lack of sensitivity in the assay used. Nevertheless, similarly to antiandrogen-treated CR-VCaP xenografts, the patients treated with a combination of GnRH agonist or GnRH antagonist and bicalutamide had a comparable intratumoral androgen milieu, with decreased serum T levels and androgen blockade. A decline in intratumoral DHT levels was observed in samples obtained from primary prostate cancer, suggesting that the DHT decline was not necessarily related to castration-resistant progression.

Overall, AR blockade altered androgen metabolism in the orthotopic VCaP xenograft model by yet unidentified mechanisms. Further studies are needed to elucidate how the results translate to clinical CRPC.

6.4 Androgen levels in primary prostate cancer patients

6.4.1 Intraprostatic androgen levels in primary prostate cancer

Serum androgen levels, particularly low testosterone, have been associated with an increased risk of prostate cancer and a high Gleason score (Morgentaler et al., 1996; Schatzl et al., 2001; Dai et al., 2012; Mearini et al., 2013; Pichon et al., 2015). Because low testosterone levels are linked to prostate cancer, it was interesting to study how circulating androgen levels reflect intraprostatic concentrations of androgens. The roles of intraprostatic testosterone and DHT in benign prostate tissue and prostate cancer are unclear, as numerous studies on intraprostatic androgens have provided inconsistent results (van der Sluis et al.,

2012). In addition, most of these studies have been based on radioimmunoassay (RIA) measurements, which are problematic due to cross reactivity and low specificity (Taieb et al., 2003). LC-MS/MS and GC-MS/MS methods providing more reliable measurements and higher specificity are expected to provide new insights for understanding the importance of intraprostatic androgen levels.

In our study, intraprostatic concentrations of DHEA, A-dione, T and DHT were measured from 48 primary prostate cancer patients using a GC-MS/MS. The results demonstrated high variability of intraprostatic androgen levels among the study cohort. Differences in the ratio of epithelial and stromal tissue possibly contributed to the high variability. Excluding the high DHT levels in cancerous samples, no significant differences in the intraprostatic androgen levels were observed between the cancerous and benign prostate samples. Similarly, Ji et al. reported that DHT levels were higher in prostate cancer samples than in paired benign prostate samples (Ji et al., 2007). Moreover, intraprostatic DHT levels were reported higher in prostate cancer samples than in benign prostate hyperplasia samples obtained from different patients (Heracek et al., 2007).

6.4.2 The effects of hormonal treatments on intraprostatic androgen levels

The patients who received hormonal treatments presented with radical changes in intraprostatic T and DHT levels. As demonstrated by other studies as well (Andriole et al., 2004; Gleave et al., 2006; Rittmaster et al., 2008), intraprostatic DHT levels were significantly lower in patients treated with 5α-reductase inhibitors (finasteride or dutasteride). *Vice versa*, intraprostatic T levels were dramatically increased in these patients. Analogous alterations were observed in both cancerous and benign prostate tissue samples. However, a few patients showed intraprostatic T and DHT levels comparable to those of non-treated patients, raising the question regarding whether they had taken 5α-reductase inhibitors, although they reported so, or whether they had a different drug response. Moreover, a patient treated with GnRH antagonist (degarelix) showed castrate levels of intraprostatic androgen levels both in serum and prostate tissue samples, indicating that ADT was successfully accomplished. Taken together, the results validate the performance of the GC-MS/MS method in practice, demonstrating that the method can be used to detect significant changes in androgen concentrations in both prostate tissue and serum samples obtained from prostate cancer patients.

6.5 The AR and AR splice variants in the VCaP xenograft model

The mRNA levels of AR-FL and AR splice variants were analyzed at different time points of the VCaP xenograft studies. Immediately after castration, the mRNA expression of AR-FL and AR splice variants V1 and V7 was up-regulated in the VCaP tumors. The AR-FL and AR splice variant expression levels were further increased in the CR-VCaP tumors, and antiandrogens enzalutamide and ARN-509 up-regulated their expression even further compared to the levels measured for the vehicle-treated CR-VCaP tumors. Similarly to our findings, it was recently demonstrated that enzalutamide and ARN-509 treatments up-regulated the mRNA expression of AR-FL and AR-V7 both in VCaP cells *in vitro* and in castrated mice with subcutaneous VCaP tumors (Asangani et al., 2016).

Because AR splice variant expression was induced by castration and the antiandrogen treatments, the gene expression of several AR splicing factors was studied. Recently, two novel RNA splicing enhancers U2AF65 and ASF/SF2 were identified to splice the pre-mRNA of AR into AR-V7 (Liu et al., 2014). We observed no differences in the mRNA expression of U2AF65 and ASF/SF2 between vehicle-treated and antiandrogen-treated CR-VCaP tumors. Other genes involved in AR splicing were also studied, but no significant changes in the gene expression were found. Thus, AR splicing was unlikely to have been altered because of antiandrogen treatment, suggesting that the expression of the AR-FL and AR splice variants is induced by an identical mechanism. The relative expression levels of AR-FL and AR splice variants were similar throughout the VCaP studies, supporting the hypothesis that the AR splicing machinery is not altered by antiandrogen treatment. Hence, the AR splice variants might only be by-products of regular AR splicing in CR-VCaP tumors. However, the variants might contribute significantly to AR signaling.

Several studies have demonstrated that AR splice variant expression in CRPC tumors is modest compared with expression levels of AR-FL (Zhang et al., 2011; Lu et al., 2015b). This finding is in line with our studies carried out using the VCaP xenograft model, which demonstrated that AR-FL was the most dominantly expressed AR form regardless of castration and antiandrogen treatment. However, new insights into the interplay between AR-FL and AR splice variants suggest that they can co-operate. AR-V7 and AR-FL can co-occupy the promoter regions of canonical androgen-responsive genes, independently of androgen or enzalutamide, but AR-V7 can bind to the promoter regions of AR-V specific targets in the absence of AR-FL (Cao et al., 2014). Remarkably, AR-Vs can promote the nuclear localization of AR-FL, even without androgens, and thus impair AR blocking by enzalutamide (Cao et al.,

2014). In addition, it was recently demonstrated that AR-Vs and AR-FL can form heterodimers with each other in an androgen-independent manner (Xu et al., 2015). It is possible that AR-FL/AR-V heterodimers can act in constitutively active manner, also in the presence of antiandrogen. Moreover, the AR-FL/AR-V heterodimer can be translocated into the nucleus without any androgens present (Sun et al., 2010). The AR-Vs could also recruit different coregulatory proteins because of their truncated protein structure, leading to an alternative non-canonical transcriptional signature. Thus, even minor changes in the expression of AR splice variants could possibly maintain AR signaling and lead to antiandrogen drug resistance in castration-resistant tumors, also in the absence of androgens.

Our results suggest that the up-regulation of AR-FL and AR splice variants may be one of the mechanisms leading to the loss of antiandrogen response after the 4-week treatment period, which is in line with the studies associating AR splice variant expression with androgen-targeted therapy resistance (Mostaghel et al., 2011; Li et al., 2013; Antonarakis et al., 2014). Most likely, a combination of different biologic alterations related to AR action enables the growth of CRPC tumors in the presence of an antiandrogen. Indeed, the latest studies underline the wide heterogeneity of AR splice variant expression in CRPC tumors, which is associated with abiraterone and enzalutamide treatment outcomes (Henzler et al., 2016; Qu et al., 2016; De Laere et al., 2017).

6.6 Novel androgenic ligands

Non-significant changes in the expression of classical androgen-regulated genes such as TMPRSS2 and KLK3 indicate that AR signaling is maintained in antiandrogen-treated VCaP tumors. As testosterone and DHT concentrations are reduced in antiandrogen-treated CR-VCaP tumors, this observation raises the question regarding whether there are other androgenic ligands present that could retain androgen-regulated gene expression, and thus, androgen-regulated VCaP tumor growth.

A few studies suggest that certain androgen biosynthesis intermediates and precursors could also activate androgen signaling by direct AR binding. Both progesterone and pregnenolone induced the growth of VCaP cells at supraphysiological concentrations and the translocalization of AR in Hep3B cells *in vitro* (Kumagai et al., 2013b). Furthermore, CYP17A1 inhibitor orteronel (TAK-700) could not block progesterone- or pregnenolone-induced cell growth or androgen-regulated gene expression in VCaP cells, suggesting that pregnenolone and progesterone can directly interact with wild-type AR and

regulate AR target gene expression without being converted to T and DHT (Kumagai et al., 2013b). Pregnenolone also stimulated LNCaP cell growth *in vitro* at physiological concentrations, comparable to DHT via mutated AR (Grigoryev et al., 2000). Interestingly, we observed remarkable concentrations of pregnenolone and progesterone in vehicle-treated and antiandrogen-treated CR-VCaP tumors. Antiandrogens had no effect on intratumoral pregnenolone levels, and progesterone levels were only decreased by the enzalutamide treatment. Thus, pregnenolone and progesterone could, to some extent, compensate for the reduced levels of intratumoral androgens in the CR-VCaP tumors. On the other hand, although intratumoral DHT levels were reduced nearly 10-fold in the antiandrogen CR-VCaP tumors, the DHT concentrations were still comparable to intratumoral pregnenolone and progesterone levels. Therefore, the residual DHT present in the antiandrogen-treated CR-VCaP tumors most likely drove the remaining androgen activity.

Whereas T and DHT are primary androgens mediating androgenic effects in normal physiological conditions, recent evidence proposes that 11-oxygenated androgens produced in the adrenals play a clinically significant role in various diseases (Turcu & Auchus, 2017). 11-oxygenated androgens 11KT and 11KDHT have been hypothesized to play an important role in CRPC, particularly in patients with low serum T levels because of ADT (Pretorius et al., 2016b). Thus, if present, 11KT, 11KDHT or other 11-oxygenated androgens would likely retain AR signaling in the CR-VCaP tumors even in the absence of intratumoral T and DHT. Pretorius et al. have demonstrated that 11KT and 11KDHT induce androgen-regulated gene expression changes and promote cell proliferation in VCaP cells (Pretorius et al., 2016a). This observation emphasizes the role that 11-oxygenated androgens may play in VCaP tumors. However, based on RNA-seq analyses, CYP11B1, HSD11B1 and HSD11B2 were not expressed in the vehicle-treated or antiandrogen-treated CR-VCaP tumors, suggesting that the conversion from the classical androgens to their 11-oxygenated androgens is unlikely under the studied conditions. Nonetheless, the precursors for 11KT and 11KDHT could be produced in the adrenal glands, secreted into the circulation and later converted to active 11-oxygenated metabolites in the CR-VCaP tumors.

6.7 AR target genes in VCaP tumors

Using genome-wide transcriptome analyses, we demonstrated that the expression levels of androgen-regulated genes were rapidly reduced in VCaP xenografts by castration. Nevertheless, androgen-regulated gene expression was restored to the pre-castration levels in the CR-VCaP tumors, indicating that the total load of AR

signaling was not altered compared with that observed in the VCaP tumors collected from intact mice. This transient effect was clearly visualized by an unsupervised hierarchical clustering of microarray data, indicating the similar expression patterns of classical androgen-regulated genes such as KLK2, KLK3 and FKBP5 as well as many non-classical androgen-regulated genes. In a similar fashion, the intratumoral androgen concentrations of T and DHT were transiently reduced one day after castration but restored in the CR-VCaP tumors. In line with these findings, ADT reduced intraprostatic androgen levels and AR target gene expression in localized prostate cancer patients but only for a short period, as expression levels remain unchanged 9 months after the initiation of treatment (Mostaghel et al., 2007). Hence, it is plausible that androgen-regulated expression is primarily driven by intratumoral androgen concentrations in CR-VCaP tumors, but not necessarily by the excessive up-regulation of AR.

By blocking the AR, antiandrogen treatment alters AR target gene expression radically. The activity of AR-FL can be blocked by antiandrogens, whereas the AR splice variants are not affected as they lack the LBD. Studies have elucidated that AR splice variants can regulate a distinct set of genes independently of AR-FL mediated gene regulation (Hu et al., 2012; Lu et al., 2015a). Therefore, the AR splice variants might have a major effect on the AR target gene signature, particularly in antiandrogen-treated CR-VCaP tumors in which AR-V expression levels are relatively high. The expression of UBE2C in particular has been associated with AR-V specific gene regulation (Wang et al., 2009; Cao et al., 2014). In our studies, we did not detect any changes in UBE2C expression despite the altered AR splice variant levels.

Moreover, we observed that the expression of two genes, NOV and ST6GalNAc1, was tremendously altered by enzalutamide and ARN-509 treatments, as they were the most up-regulated (NOV) and the most down-regulated (ST6GalNAc1) genes according to a global transcriptome analysis. The gene expression of NOV is directly regulated by the AR, causing NOV down-regulation in prostate cancer because of hyperactive AR signaling (Wu et al., 2014). Consequently, AR suppresses the expression of NOV, which inhibits the growth of prostate cancer cells (Wu et al., 2014). ST6GalNAc1 was recently demonstrated to be up-regulated in prostate cancer. As a direct AR target gene, ST6GalNAc1 expression was induced by androgen stimulation, increasing motility and decreasing adhesion in prostate cancer cells (Munkley et al., 2015). Our results suggest that NOV and ST6GalNAc1 are potential biomarkers for evaluating antiandrogen treatment responses in CRPC.

6.8 TMPRSS2-ERG gene fusions

The possible connection between AR signaling and TMPRSS2-ERG fusions has been investigated in several studies. Remarkably, it has been confirmed that ERG directly interacts with the AR. ERG expression modulates the AR cistrome, regulating the AR transcriptome in prostate cancer with PTEN loss (Chen et al., 2013). Furthermore, AR-mediated ERG expression disrupts AR signaling by inhibiting AR expression (Yu et al., 2010). Androgen-stimulated signaling via AR induces proximity of the TMPRSS2 and ERG genomic loci, and together with gamma irradiation exposure, causes DNA double-strand breaks that lead to TMPRSS2-ERG gene fusions in LNCaP cells (Mani et al., 2009; Lin et al., 2009). TMPRSS2-ERG fusions have been linked to a co-recruitment of AR and topoisomerase II beta (TOP2B), promoting TOP2B-mediated double-strand breaks in prostate cells in an androgen-regulated manner (Haffner et al., 2010).

Our RNA-seq analyses demonstrated that the primary prostate patients with TMPRSS2-ERG positive tumors had a distinct AR target gene signature compared with TMPRSS2-ERG negative tumors. Numerous androgen-regulated genes, such as NKX3.1, STEAP4 and SPOCK1, were significantly up-regulated, indicating an amplified AR signaling in TMPRSS2-ERG positive tumors. Both STEAP4 and SPOCK1 have been reported to be up-regulated in prostate cancer (Korkmaz et al., 2005; Wlazlinski et al., 2007). In contrast to our findings that NKX3.1 is up-regulated in TMPRSS2-ERG positive tumors, the loss of tumor suppressor NKX3.1 has been linked to TMPRSS2-ERG expression (Thangapazham et al., 2014; Bowen et al., 2015). Our steroid analyses confirmed that TMPRSS2-ERG positive tumors had different intratumoral androgen profiles and higher DHT/T ratios than those observed in TMPRSS2-ERG negative tumors. The results are ambiguous but raise the question concerning whether increased AR signaling exposes tumor tissue to TMPRSS2-ERG rearrangements via AR-induced proximity.

Unexpectedly, TMPRSS2-ERG fusion has been suggested to be involved in androgen biosynthesis and metabolism. Using a short hairpin RNA knockdown of ERG, it was recently demonstrated that TMPRSS2-ERG fusion regulates the activity and expression of steroidogenic enzymes in VCaP cells, including AKR1C3 and HSD17B6, whose expression levels are altered in our VCaP xenograft model (Powell et al., 2015). By direct binding to the AKR1C3 gene, ERG stimulates AKR1C3 expression, which leads to an ERG-AKR1C3-AR feed-forward loop that regulates AR signaling (Powell et al., 2015). Our results demonstrate that ERG expression remains stable in castration-resistant and antiandrogen-treated VCaP tumors, showing no evidence that ERG itself would be the cause of altered steroidogenic enzyme expression in these VCaP tumors.

However, we observed that several steroidogenic enzymes, such as HSD17B6, were differentially expressed between the ERG+ and ERG- tumor samples, supporting the possible direct involvement of ERG in the gene expression regulation of certain steroidogenic enzymes. In addition, Powell et al. reported that AKR1C3 and ERG were co-expressed in metastatic prostate cancer samples and associated with lower overall survival (Powell et al., 2015). In contrast to these findings, we observed no correlation between AKR1C3 and ERG expression in primary prostate cancer samples. An explanation for this dissimilarity could be that AKR1C3 expression is regulated differently in metastatic prostate cancer samples because of androgen-targeted treatments.

7 CONCLUSIONS

An orthotopic VCaP model was successfully established and applied to study the mechanisms of intratumoral androgen biosynthesis and the effects of novel CRPC therapies *in vivo*. Despite the castrate levels of androgens in serum, significant levels of testosterone and DHT were observed in CR-VCaP tumors using LC-MS/MS and GC-MS/MS methods, indicating an induction of intratumoral androgen biosynthesis in VCaP xenografts. The gene expression of critical steroidogenic enzymes, such as AKR1C3 and HSD17B6, was increased in the CR-VCaP tumors. Both of these enzymes are attractive drug targets for novel CRPC therapies blocking intratumoral androgen biosynthesis. Furthermore, we observed a prominent up-regulation of both full-length AR and AR splice variants in response to androgen deprivation therapy (castration) and antiandrogen treatments, identified as one of the plausible mechanisms of antiandrogen therapy resistance. Additionally, the matching approach developed using VCaP xenografts improved the experimental design and statistical power of the VCaP model, and the method can be applied to other preclinical models as well. Taken together, the VCaP model exhibits the main characteristics of clinical CRPC.

The VCaP model responded to novel antiandrogen therapies, and antiandrogen treatment with enzalutamide or ARN-509 significantly reduced the intratumoral testosterone and DHT concentrations in the CR-VCaP tumors. However, the exact mechanisms governing how antiandrogens reduce intratumoral androgen levels are unclear, and the translation of these findings to clinical CRPC remains to be confirmed. The antiandrogens also altered the gene expression of multiple steroidogenic enzymes, including many enzymes whose role in androgen metabolism has not yet been characterized. In addition, NOV and ST6GalNAc1 expression levels were altered in response to antiandrogen treatments, suggesting that they might be useful biomarkers for determining the efficacy of novel antiandrogen treatments.

The intraprostatic levels of androgens were determined in primary prostate cancer patients. The results validated the differences between cancerous and benign prostate samples with higher intratumoral DHT levels in the tumors. The study also underlined the variation in intraprostatic androgen concentrations between patients. The TMPRSS2-ERG status of prostate cancer patients was associated with distinct profiles of intratumoral androgens and altered AR target gene expression.

In general, this thesis provides new insights into and valuable information about the role of intratumoral androgen biosynthesis in prostate cancer.

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