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ANDROGEN RECEPTOR RELATED THERAPY RESISTANCE IN PROSTATE CANCER

From the Disease Model to the Mechanisms

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To my family and to memory of my parents

UNIVERSITY OF TURKU
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ABSTRACT

Androgens act through the androgen receptor (AR) and are critical regulators of prostate differentiation and function, as well as prostate cancer (PCa) growth and survival. The development of treatment resistance in castration-resistant prostate cancer (CRPC) is a major clinical concern in PCa. Although several mechanisms contribute to the treatment resistance, the AR remains a key factor in progression of the disease.

In this study, VCaP xenograft bearing mice were surgically operated by performing orchiectomy (ORX) and/or adrenalectomy (ADX) and treated with antiandrogen to model the different hormonal therapies used. Until now, it has been assumed that, unlike human, the murine adrenal cortex does not produce androgens. Our data, however, clearly indicates a contribution of the mouse adrenal gland to the intratumoral steroids in the castration-resistant VCaP tumors and to their androgen dependent growth. This is significant as it demonstrates a similarity between rodents and humans and indicates that the data obtained from the mouse models likely translate to humans better than previously anticipated.

Using VCaP tumor xenografts, we have demonstrated that the antiandrogen (enzalutamide) first stabilized tumor size, decreased serum PSA levels, and reduced intratumoral androgens, but after the treatment resistance, tumors continued to grow and intratumoral testosterone and DHT elevated back to the level observed in non-treated castration-resistant tumors. Interestingly, the tumor growth rate after ADX was slower in comparison with tumors with enzalutamide treatment and no up-regulation of DHT was observed. In summary, the data suggest that antiandrogen therapy resistance in the VCaP xenografts is associated with high intratumoral DHT production and active androgen action. Consequently, resistance to hormonal treatments is mediated by continuous activation of the AR signaling pathway caused by e.g., increased expression of the AR, AR splice variants and reactivation of tumor androgen synthesis, which together ultimately leads to DHT-mediated receptor activation.

KEYWORDS: androgens, castration-resistant prostate cancer, xenograft

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TIIVISTELMÄ

Eturauhasen kasvu ja toiminta ovat riippuvaista androgeeneista eli miessukupuolihormoneista, joita tuotetaan kivesten lisäksi pieniä määriä lisämunuaisissa. Androgeenit vaikuttavat kudoksissa androgeenireseptorin (AR) kautta ja ne ovat välttämättömiä elimistön normaaleille toiminnoille, mutta myös eturauhassyövän kasvulle. Edenneen eturauhassyövän ensisijainen hoitomuoto onkin jo vuosikymmenien ajan ollut kemiallinen tai kirurginen kastraatio, mutta ajan mittaan vaste hoidolle usein häviää ja syöpä muuttuu kastraatioresistentiksi eturauhassyöväksi. Resistenssin kehittyminen on merkittävä kliininen ongelma eturauhassyövän hoidossa. Vaikka sen taustalla on useita mekanismeja, AR ja androgeenit ovat edelleen tärkeitä tekijöitä taudin etenemisessä.

Tässä tutkimuksessa hiiriin inokuloitiin ihmisen VCaP syöpäsoluja ja muodostuneita kasvaimia hoidettiin kivesten ja lisämunuaisten poistolla sekä antiandrogeenilla hormonaalisten hoitojen mallintamiseksi. Vastoin aikaisempaa yleistä olettamusta tutkimuksemme osoittaa, että hiiren lisämunuaisilla on vaikutusta kasvaimen androgeenipitoisuuksiin ja kasvuun. Havainto on tärkeä, sillä se osoittaa, että hiirimalleilla saadut tulokset ovat paremmin verrattavissa potilaiden tilaan.

VCaP ksenografti-tutkimusmallilla olemme osoittaneet antiandrogeenin (entsalutamidin) hidastavan kasvaimen kasvua, vähentävän seerumin PSA pitoisuutta ja kasvaimen androgeeni pitoisuuksia väliaikaisesti, mutta hoitoresistenssin muodostuttua kasvaimen testosteroni sekä DHT pitoisuudet nousevat hoitoa edeltävälle tasolle ja kasvaimet jatkavat kasvuaan. Mielenkiintoista on, että kastraation ja lisämunuaisten poiston yhdistelmällä, vastaavaa androgeenien nousua ei kasvaimissa havaittu ja niiden kasvu oli hitaampaa. Yhteenvetona tulokset viittaavat siihen, että resistenssi hormonaalisille hoidoille välittyy jatkuvan AR signalointireitin kautta, joka AR-reseptorin muutosten sekä kasvaimen androgeenisynteesin aktivoitumisen vuoksi johtavat DHT-välitteiseen reseptoriaktivaatioon.

AVAINSANAT: androgeenit, kastraatioresistentti eturauhassyöpä, ksenografti

Table of Contents

Abbreviations

ACTH	Adrenocorticotropin
A-dione	Androstenedione
ADT	Androgen deprivation therapy
ADX	Adrenalectomy; surgical removal of the adrenal glands
AKR1C3/HSD17B5	Aldo-Keto Reductase Family 1 Member C3
AR	Androgen receptor
ARE	Androgen-response element
AR-FL	Full-length androgen receptor
AR-Vs	Androgen receptor splice variants
BPH	Benign prostatic hyperplasia
CRH	Corticotropin-releasing hormone
CRPC	Castration-resistant prostate cancer
ctDNA	Circulating tumor DNA
CYP11A1	Cytochrome P450 Family 11 Subfamily A Member 1
CYP17A1	Cytochrome P450 Family 17 Subfamily A Member 1
de novo	From the beginning
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
DHT	Dihydrotestosterone
E2	Estradiol
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptor
HSP90	Heat shock protein 90
in vitro	Outside a living organism
in vivo	In a living organism
LH	Luteinizing hormone
LHR, Lhcgr	Luteinizing hormone receptor
LHRH	Luteinizing hormone-releasing hormone
LNCaP	Lymph node metastasis prostate cancer

mCRPC	Metastatic castration-resistant prostate cancer
mHSPC	Metastatic hormone-sensitive prostate cancer
MR	Mineralocorticoid receptor
NEPC	Neuroendocrine prostate cancer
nmCRPC	Non-metastatic castration-resistant prostate cancer
NOV	Nephroblastoma overexpressed
o.t.	Orthotopic, orthotopically
ORX	Orchiectomy; Surgical castration
OS	Overall survival
P_4	Progesterone
PCa	Prostate cancer
PR	Progesterone receptor
PSA	Prostate-specific antigen
PSMA	Prostate specific membrane antigen
RNA-seq	RNA-Sequencing
s.c.	subcutaneous, subcutaneously
SRD5A1/2	Steroid 5 Alpha-Reductase 1/2
T	Testosterone
TMPRSS2	Transmembrane serine protease 2
t-NEPC	therapy-induced neuroendocrine prostate cancer
VCaP	Vertebral prostate cancer

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Huhtaniemi R, Oksala R, Knuuttila M, Mehmood A, Aho E, Laajala TD, Nicorici D, Aittokallio T, Laiho A, Elo L, Ohlsson C, Kallio P, Mäkelä S, Mustonen MVJ, Sipilä P, Poutanen M. Adrenals Contribute to Growth of Castration-Resistant VCaP Prostate Cancer Xenografts. Am J Pathol. 2018 Dec;188(12):2890–2901.

II Huhtaniemi R, Sipilä P, Junnila A, Oksala R, Knuuttila M, Mehmood A, Aho E, Laajala TD, Aittokallio T, Laiho A, Elo L, Ohlsson C, Thulin MH, Kallio P, Mäkelä S, Mustonen MVJ, Poutanen M. High intratumoral dihydrotestosterone is associated with antiandrogen resistance in VCaP prostate cancer xenografts in castrated mice. iScience. 2022 Apr 25;25(5):104287.

1 Introduction

Cancer incidence and mortality are growing fast worldwide (Bray et al., 2018). This is largely due to aging and the growth of the population, as well as changes in the prevalence and distribution of the main risk factors for cancer, several of which are associated with socioeconomic development such as life expectancy, education and level of wealth (Bray et al., 2012). According to the World Health Organization, prostate cancer (PCa) is the second most common cancer and the fifth leading cause of death due to cancer in men worldwide (Bray et al., 2018). In the Nordic countries, the lifetime risk of getting PCa is almost 19 percent, meaning that every fifth man will be diagnosed with PCa before the age of eighty-five (Danckert et al., 2019). Despite the early diagnosis and surgical and medical treatments in about 20% to 30% of patients PCa metastasize (Han et al., 2003) and disease progression will eventually lead to death.

Androgens (male sex hormones) act through the androgen receptor (AR) and are critical regulators of prostate differentiation and function, as well as PCa growth and survival via binding to androgens. AR regulates several distinct functions of prostate cells, including the proliferation, differentiation, survival, apoptosis and protein synthesis of the prostate-specific antigen (PSA), a clinically used biomarker for PCa.

Androgen deprivation therapy (ADT) reduces androgen production by surgical or chemical castration, and it is the main and most effective initial treatment of PCa. However, according to increasing PSA levels, PCa typically becomes resistant to androgen ablation within a median of 14–20 months (Crawford et al., 1989; Eisenberger et al., 1998) and the disease progresses to castration-resistant prostate cancer (CRPC). Before 2010, the taxane based cytotoxic drug docetaxel was the first and only life-prolonging agent for metastatic CRPC (mCRPC) (Petrylak et al., 2004; Tannock et al., 2004). In recent years, the survival of patients with CRPC has improved with the use of two new generation hormonal therapies, such as antiandrogens (Beer et al., 2014; Scher et al., 2012) and an androgen biosynthesis inhibitor, abiraterone (de Bono et al., 2011; Ryan et al., 2013). However, all the patients are not responsive to the treatments and develop resistance to these drugs in a short time, probably due to both activation of AR-dependent pathways and the mechanisms independent of AR signaling.

The present thesis focuses on the antiandrogen resistance and mechanism of the steroid hormone action on a preclinical *in vivo* VCaP xenograft model of CRPC. The *in vivo* animal model is based on the subcutaneous (*s.c.*) inoculations of well-characterized VCaP cells, originating from human vertebral PCa (Korenchuk et al., 2001), to the immunodeficient mice. However, in contrast to human, murine adrenals do not produce large quantities of dehydroepiandrosterone (DHEA) or its sulphate (DHEA-S) and according to a previous view nor androgens (van Weerden et al., 1992), and thus, the value of preclinical mouse models for studying androgen dependency of the CRPC has been questioned. One of our aims has been to challenge this dogma, and to study CRPC and to define antiandrogen resistance mechanisms *in vivo*. The key techniques in our study were the measurement of intratumoral, intra-adrenal and serum steroid [progesterone (P_4), androstenedione (A-dione), testosterone (T) and dihydrotestosterone (DHT)] concentrations and global gene expression profiling with RNA-Sequencing (RNA-seq). Using these methods, we have established that, VCaP xenografts respond to enzalutamide, indicating the value of the model in studying androgen dependence on CRPC. Novel finding showed that, mouse adrenals do produce steroids and precursors for intratumoral androgen synthesis and finally, antiandrogen resistance is associated with the re-activation of DHT synthesis in the tumors. The results of our research prove importance of the AR role in PCa, and that the AR pathway can be studied with this model system. Most importantly, these efforts will provide knowledge to help develop AR targeting therapies and new treatment options for men with PCa.

2 Review of the Literature

2.1 Prostate cancer

The Prostate is an exocrine gland of the male reproductive system located around the urethra, near the bladder and its main function is to secrete nourishing and protective fluid to the semen. Prostate related diseases have a great impact on men's health and morbidity manifested as benign prostatic hyperplasia (BPH) (the enlargement of the prostate), prostatitis (the inflammation of the prostate) and PCa (Roehrborn, 2006). The prostate epithelium consists of basal, intermediate, luminal, and neuroendocrine cells. Over 90% of PCa are acinar adenocarcinomas raised from luminal cells and the rest are non-acinar carcinoma variants or types like ductal carcinoma, basal cell carcinoma and neuroendocrine tumors (Baig et al., 2015; Humphrey, 2012). Because acinar adenocarcinomas are the most common form, PCa generally refers to androgen-dependent acinar adenocarcinomas. PCa is often multifocal and occurs in several separate spots. About 70% of PCa cases are found in the peripheral zone, 20% in the transition zone and 10% in the central zone of the prostate (Lavery et al., 2016).

PCa is the second most common cancer for men after lung cancer and the fifth leading cause of death worldwide with a higher prevalence in developed countries (Bray et al., 2018). The risk of developing PCa increases with aging. Over 1.2 million new cases of PCa were reported worldwide in 2018 and incidence and mortality rates of PCa are strongly related to the age, with the highest incidence being seen in over 65-year-old men (Bray et al., 2018). Overall survival (OS) has improved over the last few decades, likely due to early diagnosis and advanced treatment modalities. Despite the effectiveness of new therapies, in about 20 to 30% of the patients the disease progress to the lethal form of the disease, known as castration-resistant PCa (CRPC) (Han et al., 2003).

The PSA, also called kallikrein 3, is a glycoprotein produced in prostate ductal and acinar cells of prostate epithelium. As a protease, its functions are to cleave the seamen proteins to smaller polypeptides and to keep it in a more liquid form, promote sperm motility, dissolve cervical mucus and prolong the lifespan of spermatozoa by neutralizing vaginal alkalinity (Lilja, 1997; Robert et al., 1997). PSA is secreted mainly into the semen, but also small amounts are found in the bloodstream. Blood

levels of PSA are used for diagnosis and cancer progression monitoring though PSA could be increased in addition to PCa in BPH and prostatitis (Nadler et al., 1995). At the early stage PCa is often asymptomatic, slow growing and needs only minimal or no treatment. The first symptoms are increased urination frequency and excessive urination at night, which can also indicate benign prostatic hyperplasia. At a more advanced stage, the most common symptoms are urinary retention and bone metastases related back pain. Bone is the most common organ site where PCa spreads in addition to lungs, liver, and lymph modes (Halabi et al., 2016).

2.1.1 Epidemiology

PCa incidence and mortality rates are highly variable worldwide and the reasons for these differences among the countries are not fully clear, although it might be attributed to increased PSA testing and increasing life expectancy (Bray et al., 2018). Although population-based PSA screening reduces PCa mortality, it is not recommended because it may lead to the over diagnosis of slow growing tumors and overtreatments (Parker et al., 2015). The incidence and mortality of PCa worldwide correlates with increasing age, with the average age at the time of diagnosis being 66 years. PCa is rarely lethal in localized or regional disease (5-year survival, nearly 100%), but prognosis is worse in metastatic disease (5-year survival 31%) (Howlader et al., 2018).

The etiology of PCa remains unknown in comparison with the other common cancers. PCa is a clinically heterogeneous disease. Whereas some men have an aggressive form of PCa, most others have a slow growing or indolent form of disease, and the risk factors differ markedly for potentially lethal and indolent disease (Jahn et al., 2015). The main risk factors are advanced age, ethnicity, genetic factors, and family history (Bostwick et al., 2004; Hemminki, 2012; Pienta & Esper, 1993). Other factors that are associated with the development of PCa include a high fat and red meat diet without fruit and vegetables, obesity and physical inactivity, chronic inflammation, hyperglycemia, infections, and environmental exposure to chemicals like smoking and herbicides (Markozannes et al., 2016; Mullins & Loeb, 2012; Myles et al., 2008; Vidal et al., 2014; Wolk, 2005). It is hypothesized that due to hereditary, social, and environmental factors to the incidence rates and aggressiveness of PCa are higher in African American men compared to White men and their mortality is approximately twice as high as White men (Panigrahi et al., 2019).

After Huggins and Hodges (Huggins & Hodges, 1941), a number of studies have demonstrated an association between androgens and PCa, but their role in the development of PCa has not been resolved. According to PCa prevention studies 5-α-reductase enzyme inhibitors, which prevent T conversion to more potent androgen, DHT, reduced the risk of being diagnosed with PCa (Wilt et al., 2010). However, the

prevalence of aggressive and rare types of PCa increased with 5-α-reductase inhibitors (Andriole et al., 2010; Thompson et al., 2003). But after the 18 years of follow-up, there was no difference in the rates of OS or survival, with the 5-α-reductase inhibitor group compared to the placebo group, after the diagnosis of PCa (Thompson et al., 2013).

2.1.2 Diagnosis and progression

Many PCas are detected with elevated PSA levels, but the actual diagnosis can only be made by tissue biopsy histology, being the reliable method to differentiate cancer from benign hyperplasia. As PCa is a heterogeneous disease, from small, indolent, and low-grade tumors to large, aggressive, and fatal tumors, the determination of tumor type is important for planning treatment options. Generally, a localized disease can be classified in to low, intermediate, and high-risk PCa depending on Gleason patterns, PSA level, and the clinical stage (Table 1).

PCa is usually diagnosed by a digital rectal examination (DRE), a PSA blood test and confirmed with a transrectal ultrasound (TRUS) or a magnetic resonance imaging (MRI) guided biopsy. Even though traditional TRUS biopsy analysis has been a standard analysis, it misses about 21–28% of cancers and under grade 14–17% of the cases (Bjurlin et al., 2013). MRI with real-time ultrasonography enables the visualization of suspicious lesions for a more targeted biopsy, and it has been able to find 30% more high risk cancers and 17% low-risk cancers than a traditional TRUS- guided biopsy alone (Siddiqui et al., 2015).

Table 1. Risk groups and main treatment options of nonmetastatic prostate cancer. Risk groups for localized prostate cancer are used to design treatment and in the assessment of patient prognosis. Clinical tumor stage is the extent of the main (primary) tumor (T). The Grade group, based on the histology Gleason score (GS), is a measure of how likely the cancer is to grow and spread. Prostate-specific antigen (PSA), radical prostatectomy (RP), radiation therapy (RT), androgen deprivation therapy (ADT). (Fizazi & Gillessen, 2023; Parker et al., 2020)

RISK GROUP	TUMOR TYPE	TREATMENT OPTIONS
Low risk	T1-T2a and GS ≤ 6 and PSA ≤ 10 ng/ml	Active surveillance, RP, RT
Intermediate risk	T2b and GS = 7 and/or PSA 10-20 ng/ml	RP, RT ± ADT, active surveillance
High risk	≥ T2c or GS 8-10 or PSA > 20 ng/ml	ADT + RT ± docetaxel, RP + pelvic lymphadenectomy

Diagnosis of the biopsy is based on the microscopic evaluation resulting to a Gleason grade. It describes how much the cancer from a biopsy looks like well-differentiated healthy tissue (lower score) or poorly differentiated abnormal tissue (higher score), using a scale of 1 to 5 based on the microscopic architecture and appearance of the tumor cells (Gleason, 1977). The diagnosis gives one Gleason grade to the most predominant histology pattern in the sample and another Gleason grade to the second most predominant histology pattern, then added together to determine the Gleason score (Epstein et al., 2016). PCas with a higher Gleason score are more aggressive and have a worse prognosis.

The PSA testing is used in various stages in PCa management, including screening, the evaluation of the future risk of cancer development, the detection of recurrent disease after local therapy and in the management of advanced disease. Although it is an organ specific marker, it is not cancer specific and PCa can be present without elevated PSA (Thompson et al., 2005) and on the other hand, increased PSA values from 4–10 μg/l, do not always indicate the appearance of PCa (Carter, 2004). Several laboratories determine the upper limit of normal PSA as 4 μg/l. The use of an age specific reference range for serum PSA levels has reduced unnecessary biopsies in older men, because the prostate is usually enlarged with age and it secretes more PSA (Oesterling et al., 1993). PSA is a pivotal tool for biochemical recurrence diagnosis after local treatment, like radical prostatectomy or radiation therapy, and after later therapies. It is generally accepted that PSA levels should be less than 0.2 μg/l after radical prostatectomy, and after radiation therapy below 0.5 μg/l (Ziada et al., 2000). In 20–40% of men, undergoing radical prostatectomy with local disease, serum PSA will elevate within ten years after surgery (Freedland et al., 2005; Hull et al., 2002; Roehl et al., 2004) and median time for the development of metastases following PSA elevation was eight years (Pound et al., 1999). After external-beam radiation therapy 30–50% of the patients will experience a biochemical recurrence within ten years (Kupelian et al., 2006).

Clinical staging is based on the tumor-node-metastasis (TNM) system according to how large a primary tumor is (T1-T4), has the tumor spread in the lymph nodes (N0-N1) and has the cancer spread in other parts of the body (M0-M1) (American Joint Committee on Cancer, 2010; Parker et al., 2015). The most common site to PCa metastases is bone (Halabi et al., 2016). A bone scan with radioisotope is usually the primary study of suspected bone metastases. A computed tomography (CT) scan with or without abdominal imaging is used to look for metastasis in the lymph nodes or other nearby visceral (internal) organs. However, findings should be confirmed by other imaging studies e.g., magnetic resonance imaging (MRI), X-ray or positron emission tomography (PET) scan (Cornford et al., 2017), as these standard imaging modalities have poor sensitivity and specificity at low PSA levels (Taneja, 2004).

The new prostate-specific marker, prostate-specific membrane antigen (PSMA), which has replaced almost all previously used PET markers, offers new opportunities for determining the spread of the disease and detecting recurrences with very low PSA values (Bouchelouche & Choyke, 2018).

Progression of the disease and common management options after radical prostatectomy and biochemical recurrence are presented in Figure 1. Treatment options are discussed later in this chapter.

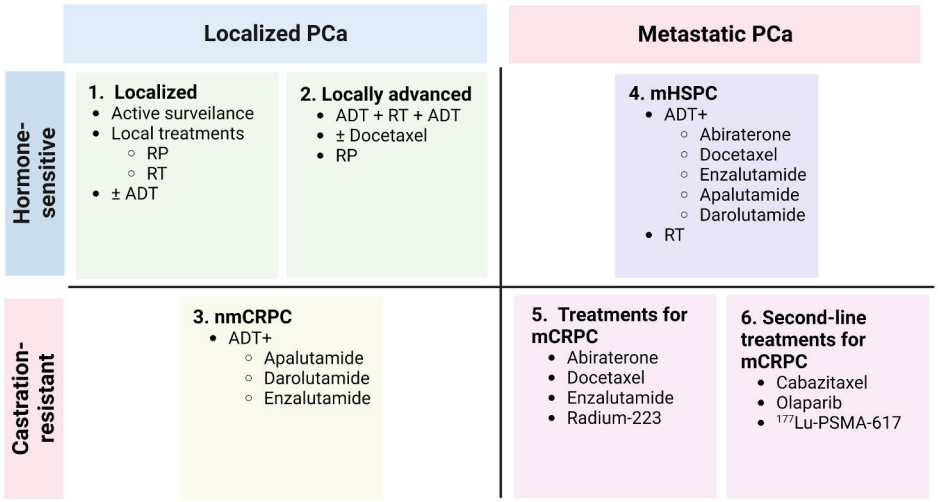

Figure 1. PCa treatment strategies for different stages (Fizazi & Gillessen, 2023; Parker et al., 2020). Radical prostatectomy (RP), radiation therapy (RT), androgen deprivation therapy (ADT), non-metastatic castration-resistant prostate cancer (nmCRPC), metastatic hormonal-sensitive prostate cancer (mHSPC), metastatic (mCRPC), Radium alpha particle-emitting radiopharmaceutical (Radium-223) and Lutetium-177 prostate-specific membrane antigen radioligand therapy (^{177}Lu-PSMA-617). Created with BioRender.com

2.2 Androgen receptor and androgen biosynthesis in prostate cancer

Androgen receptor (AR) was discovered and characterized in the late 1960s by Anderson, Bruchovsky, Mainwaring and colleagues (Anderson & Liao, 1968; Bruchovsky & Wilson, 1968; Mainwaring, 1969) based on a hypothesis that a receptor might be needed to mediate the biological effect of androgens. Androgen dependence on PCa was figured out earlier, in 1941, from the notion of the beneficial effects of castration and injection of estrogens in patients with metastatic PCa (Huggins & Hodges, 1941).

Androgens and AR are essential regulators of normal male physiology and health. AR is expressed in the cells of almost all tissues in the body (Gelmann, 2002)

and upon binding of the androgens, AR translocate from the cytoplasm into the nucleus to regulate the gene transcription and thereby the activation of target genes (Heinlein, 2004). A decline in circulating androgens has been associated with co-morbidities such as cardiac diseases (Jones et al., 2005), central obesity (Khaw & Barrett-Connor, 1992), diabetes (Ding et al., 2006) and metabolic syndrome (Kupelian et al., 2006; Laaksonen et al., 2004). The Binding of T and DHT to AR, initiates the pubertal development and differentiation, sexual life and fertility in both males and females. On the other hand, male sexual development fails without androgens or functioning AR and complete loss of AR in men results in androgen insensitivity syndrome (AIS) with impaired masculinization (Hughes et al., 2012).

Androgen synthesis is regulated by the hypothalamic-pituitary-gonadal axis. In the hypothalamus circulating androgens bind to the AR to regulate the production of a gonadotropin-releasing hormone (GnRH). Pulsatile release of GnRH stimulates the secretion of the luteinizing hormone (LH) by pituitary gland and its release into the peripheral circulation. LH binds to the LH receptors (LHR) mainly in the testes but also in the adrenal gland (Bernichtein et al., 2008; Miyazawa et al., 2017) inducing the production of androgens. Increased androgen levels decrease GnRH and LH production via negative feedback loop maintaining serum T at physiological levels (Figure 2). Similarly, corticotropin-releasing hormone (CRH) from the hypothalamus stimulates adrenocorticotropin (ACTH) release from the pituitary, which stimulates the adrenal glands to produce cortisol, DHEA, dehydroepiandrosterone sulfate (DHEA-S), A-dione, androstenediol, 11β-hydroxyandrostenedione (11OHA) and small amounts of T (Sharifi & Auchus, 2012; Turcu et al., 2014). Although these steroids have little androgenic activity, they provide a number of circulating precursors for peripheral conversion to more potent androgens such as T and DHT (Barnard et al., 2020).

Androgens are also regulators of a normal prostate as well as PCa cells. It is now obvious that androgens and AR play a pivotal role in the progression of PCa and that the majority of PCa express the AR throughout the progression of the disease (Lonergan & Tindall, 2011; Ruizeveld de Winter et al., 1994). The suppression or elimination of testicular androgen production is still the most effective therapy for metastatic PCa, but it is not curative and within two to three years remission will recur to the lethal phenotype of the disease, termed castration-resistant PCa (CRPC) (Crawford et al., 1989; Eisenberger et al., 1998). The recurrence is observed with an increasing serum PSA, increasing tumor size, new metastatic spread, and disease-related symptoms and have typically results in death within 16 to 18 months (Pienta & Bradley, 2006). However, many studies have shown that the AR is able to retain activity even in the CRPC stage through the aberrant mechanisms of activation. In fact, new androgen receptor signaling inhibitor therapies and their combinations have been shown to extend survival up to 25 months in clinical trials (Turco et al., 2022).

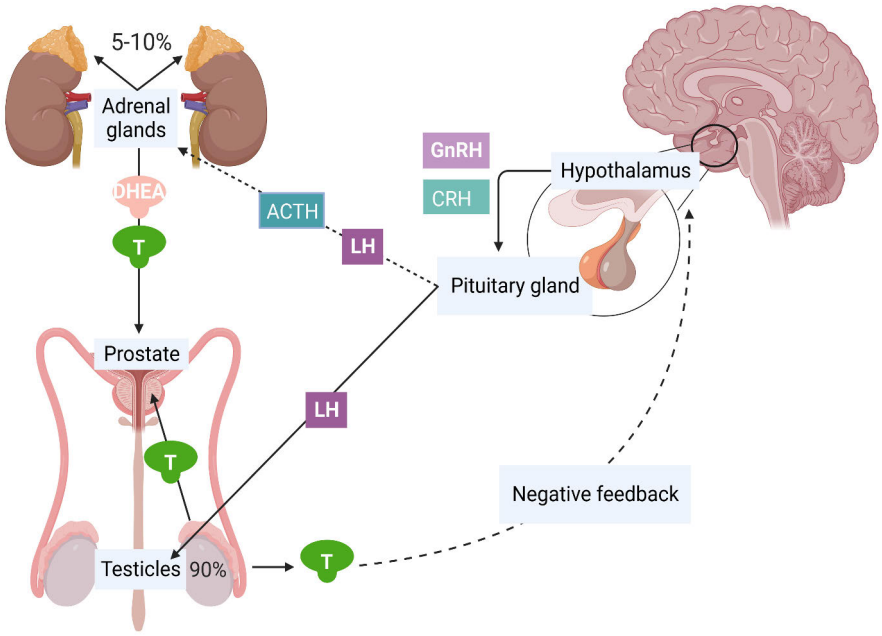

Figure 2. Regulation of androgen synthesis in men. Androgen synthesis is regulated by the hypothalamic-pituitary-gonadal axis. Pulsatile release of a hypothalamic peptide, gonadotropin-releasing hormone (GnRH), stimulates the secretion of a luteinizing hormone (LH) by pituitary gland and its release into the peripheral circulation. LH binds to the LH receptors mainly in the testes, but also in the adrenal gland and stimulates the production of androgens. This creates a high local concentration of testosterone (T) in the testes and to the secretion of the T into the circulation, being also the operator of the negative feedback loop that prevents GnRH release by the hypothalamus. Specifically, when T levels are sufficient, the pituitary gland decreases the production and release of LH, which also inhibits hypothalamic GnRH secretion. Corticotropin-releasing hormone (CRH) from the hypothalamus stimulates adrenocorticotropin (ACTH) release from the pituitary, which stimulates the adrenal glands to produce smaller proportion (about 5-10%) of androgens e.g., dehydroepiandrosterone (DHEA) and T. Created with BioRender.com.

2.2.1 AR structure and normal function

AR is a 110-kDa phosphoprotein and a member of nuclear receptor superfamily with similar steroid receptor structure to the estrogen receptors (ER), a progesterone receptor (PR), glucocorticoid receptor (GR) and a mineralocorticoid receptor (MR) (Mangelsdorf et al., 1995). The AR gene (gene name NR3C4) is located on chromosome X (Xq11-12) and consists of eight exons coding about 11 kDa mRNA (Gelmann, 2002). AR is a transcription factor with a large N-terminal transactivation domain [NTD (exon 1)], a C-terminal ligand-binding domain [LBD (exons 4–8)], a central DNA-binding domain [DBD (exons 2–3)], and a small hinge region between the DBD and LBD (Gelmann, 2002). All domains are important to receptor function. The hinge region contributes AR nuclear localization and degradation (Haelens et al.,

2007). Via the DBD domain AR binds to the promoter and enhancer region of AR regulated genes and the NTD and LBD stimulate the transcription of these AR regulated genes. The LBD is also a target for the competitive AR antagonist used in the PCa treatment (Knudsen & Scher, 2009) and its deletion and mutations can lead to AR unresponsiveness to androgens and antiandrogen treatments (Watson et al., 2015).

The unliganded AR is held inactive in the cytoplasm with an HSP90 chaperone complex, or alternatively it undergoes ubiquitin-proteasome mediated degradation in the absence of ligand (Lee & Chang, 2003; Roy et al., 2001). Normal physiologic ligands, T or DHT, bind to the LBD of AR causing conformational changes of the AR and inhibitory heat shock proteins are released. Dimerized AR protein translocate into the nucleus to exert its function. It binds to the selective androgen-response elements (AREs) within the regulatory regions of various target genes (Gelmann, 2002). AR recruits' co-regulatory proteins that are differentially expressed in different types of cells and complex binds to AREs to facilitate transcription, leading to responses such as growth and cell survival by turning on or off the target genes (Dai et al., 2017) in Figure 3.

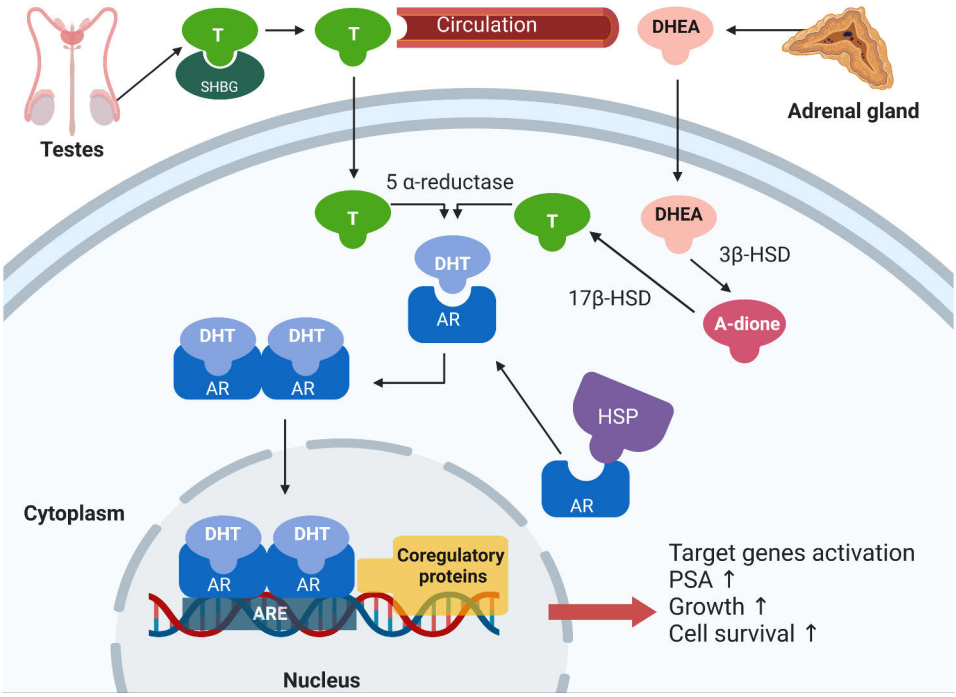

Figure 3. Androgen receptor action in prostate. Testosterone (T) bind directly to the androgen receptor (AR) in the prostate or is converted via the 5α-reductase enzymes to dihydrotestosterone (DHT). Androgen responsive elements (ARE), androstenedione (A-dione) dihydroepiandrogen (DHEA), heat shock protein (HSP), sex hormone binding globulin (SHBG). Created with BioRender.com

AR is expressed in the cells of almost all tissues in the body and its mRNA levels are regulated by androgen and by other steroid hormones (Gelmann, 2002). AR action is regulated to some extend by negative feedback via the AR gene itself indicated by the fact that castration results in increased AR mRNA that is reversed by the administration of androgen in rat ventral prostate and in a human PCa LNCaP cell line (Quarmby et al., 1990; Tan et al., 1988). Furthermore, androgens can regulate AR at the level of both mRNA and protein. Prolonged androgen treatment decreased AR mRNA levels and increased AR protein levels in LNCaP PCa cell line (Krongrad et al., 1991) and in contrast, treatment of the castrated animals with DHT restored the AR mRNA level (Takeda et al., 1991). Androgens have also been shown to increase AR protein levels in various cell lines (Wolf et al., 1993; Yeap, 1999). AR is not stable because of its relatively short half-live and is degraded without ligand (Lee & Chang, 2003) e.g., in LNCaP cells the half-life is approximately 3 h in the absence of androgens and is longer than 10 h in the presence of 10 nm DHT (Gregory et al., 2001). It should also be noted that androgens and AR have also been shown to act independently of each other in the regulation of cellular processes using non-classical and non-genomic mechanisms (Foradori et al., 2008).

2.2.2 Androgen biosynthesis

The main circulating male sex steroid, T, is synthesized primarily by Leydig cells in the testes (Hohl, 2017). Testes average secretion rate for the T is 7 mg per day, corresponding to serum concentrations of 3.8 ng/ml (Flück & Pandey, 2017; Hammond et al., 1977) Testes also release about 70 µg per day of DHT (Hammond et al., 1977; Marcelli, 2017). The majority of circulating T and DHT (97–99%) is bound to serum sex hormone binding globulin (SHBG) and albumin (Baker, 2002; Rosner et al., 1991). Thus, only 1% to 3% of androgen is in a free form that can enter the target cells through a mechanism of passive diffusion. Once T diffuses into the cell, it either binds to the AR or, it can be converted into the more active metabolite DHT by the 5α-reductase isoenzymes (SRD5A1 or A2; Steroid 5 Alpha-Reductase 1 or 2), or alternatively into estradiol (E2) by the enzyme aromatase (CYP19A1; Cytochrome P450 Family 19 Subfamily A Member 1). DHT is the principal androgen found within the prostatic cell nucleus (Bruchovsky & Wilson, 1968) and it binds to the AR with high affinity, and its biological activity is up to ten times as potent as T (Deslypere et al., 1992). Approximately 25% of circulating DHT is produced by the testis and the rest of is produced in tissue such as the prostate and skin (Imamoto et al., 2008).

While about 90% of androgens in men are produced in the testis, a small proportion is produced in the zona reticularis of the adrenal cortex. Approximately 5% of serum T is from adrenal origin (Hohl, 2017) and studies on patients with PCa have been

demonstrated that human adrenals produce T regardless of whether the patient had testes intact or was castrated (Sanford et al., 1977). However, studies have proved that the steroid concentration of the circulation is not proportional to the concentration of the human prostate tissue (Marks et al., 2006; Page et al., 2006, 2011).

The adrenal glands are also responsible for the release of large amounts of low potent androgens, such as DHEA and A-dione, to the bloodstream. It is noteworthy that circulating levels of DHEA are more than 100 times higher than T (Labrie et al., 2005), thus, providing substrates for conversion into androgens in the peripheral tissues to T and DHT, or directly to DHT by PCa cells (Mostaghel, 2013). Up to 50% of intraprostatic DHT is still present after castration and no decrease of A-dione have found in the prostate after castration (Bélanger et al., 1989; Miyamoto et al., 1998).

Through LH stimulation, the biosynthesis of all steroid hormones begins with 27-carbon cholesterol, which is gradually modified with enzymes to 21-carbon progestins and then to 19-carbon androgens (Figure 4). In the first step of steroidogenesis, cholesterol molecules are transported to the mitochondrial membrane by steroidogenic acute regulatory protein (StAR). In the conventional canonical pathway of T biosynthesis in the testis is primarily performed, either through the delta 5 pathway via pregnenolone or through the delta 4 pathway via P_4 (Flück et al., 2003). In the mitochondrial membrane side chain cleavage enzyme CYP11A1 (Cytochrome P450 Family 11 Subfamily A Member 1), convert cholesterol to pregnenolone which is further converted through the delta 5 pathway to 17α-hydroxypregnenolone (17α-OH-pregnenolone) and DHEA by the enzyme CYP17A1 (Cytochrome P450 Family 17 Subfamily A Member 1). DHEA is then converted to T via A-dione or androstenediol (5A-diol) either first by Hydroxy-Delta-5-Steroid Dehydrogenase, 3 Beta- and Steroid Delta-Isomerase 2 (HSD3B2) or Aldo-Keto Reductase Family 1 Member C3 (AKR1C3/HSD17B5). In some androgen targeted tissues, such as the prostate, T will be further converted to more potent DHT by SRD5A2 (Mostaghel, 2013). In humans, little conversion of pregnenolone to A-dione occurs in the testis through the delta 4 pathway originating from P_4 and 17α-hydroxyprogesterone (17α-OH-P_4), because of the poor activity of CYP17A1 on the substrate 17α-OH-P_4 compared with 17α-OH-Pregnenolone and because HSD3B2 activity is less abundant (Flück et al., 2003). By contrast, rodents produce T predominantly via the P_4 to A-dione delta 4 pathway (Fevold et al., 1989; Namiki et al., 1988). However, the conventional canonical androgen synthesis pathway does not provide an explanation for the androgen production in some normal and pathological conditions such as in PCa. Androgens can be also produced by alternative pathways, such as the backdoor pathway and the 5α-dione pathway, which do not use T as an intermediate to DHT production (Figure 4). Various synthesis pathways for DHT production are described in more detail later in the text.

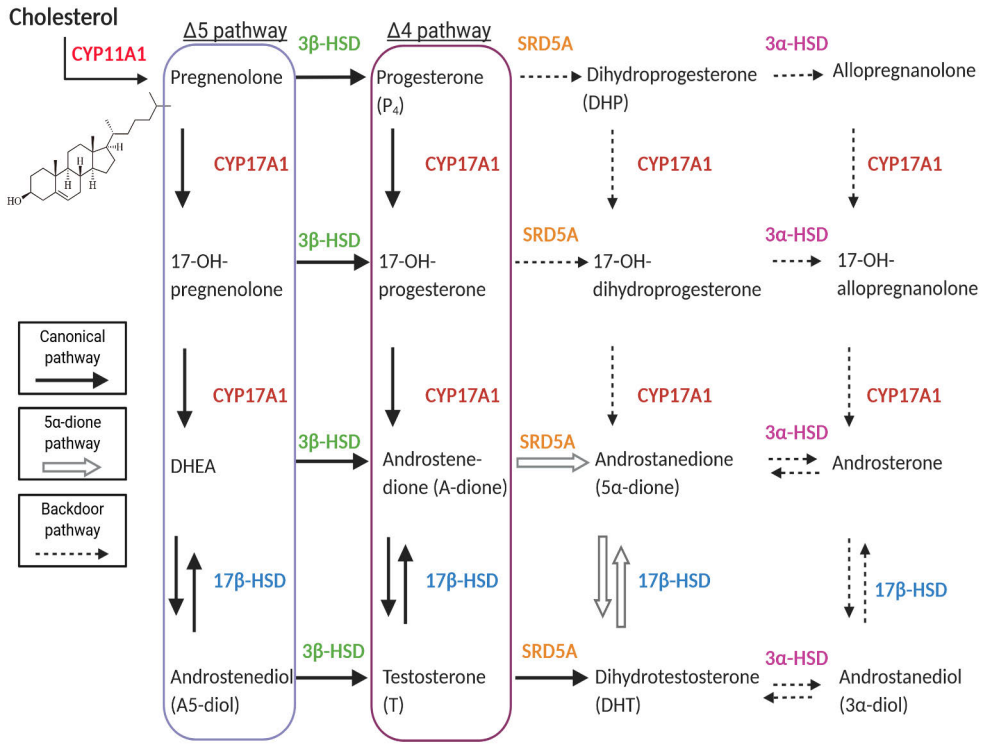

Figure 4. Androgen biosynthesis pathways. In the conventional canonical pathway of testosterone (T) biosynthesis in the testis is primarily performed, either through the delta 5 pathway via pregnenolone or through the delta 4 pathway via progesterone (P_4) Other alternative androgen synthesis pathways in addition to the classical Canonical pathway persist. The classic androgen biosynthesis pathway is shown in black arrows, the more recently described alternative pathways, the backdoor pathway in dashed arrows and the 5α-dione pathway in white arrows. Created with BioRender.com

2.3 Management of prostate cancer

PCa management is complicated as it covers the spectrum from indolent to highly aggressive disease. The survival benefits of different therapies, as well as their comparative efficacy and relative toxicity, remain controversial. Localized PCa can be curatively treated by surgical resection of the prostate or through radiation therapy (Catalona et al., 1999; Swanson, 2006). However, once metastases have developed, PCa is mostly incurable, and all therapy is palliative. Invasive or even micro metastatic disease presents a clinical challenge, as these tumors respond poorly to standard cytotoxic treatments.

Experimental and clinical evidence suggest a role for T in the etiology of PCa as the majority of prostate tumors express the AR. Therefore, the primary treatment of metastatic PCa is based on an ADT with surgical or medical castration as prostate cells are dependent on androgens for development, growth, and survival (Penning, 2015).

The ADT improves survival in most of men about 2 to 3 years until disease relapses as CRPC (Harris et al., 2009). More than 90% of men with mCRPC develop bone metastases which are later associated with an increased risk of skeletal events (SREs) with painful pathological fractures and spinal cord compression (Bubendorf et al., 2000; Klaassen et al., 2017).

After the resistance to ADT, androgen synthesis occurs in the testes (Daskivich & Oh, 2006; Raddin et al., 2011), adrenals and the tumor itself, through the activation of alternative steroid synthesis pathways. These findings have led to the development of more potent new second-line androgen deprivation therapies for the suppression of androgen actions i.e., antiandrogen enzalutamide and androgen synthesis inhibitor abiraterone. These therapies have successfully prolonged the life of patients with advanced PCa, but resistance to these drugs typically occurs within 6–18 months (Beer et al., 2014; Ryan et al., 2014). However, according to studies conducted in recent years, the AR pathway remains active even after the resistance to new AR-targeted therapies enzalutamide and abiraterone (Li et al., 2013; Mostaghel et al., 2011) and these AR-targeted therapies usually maintain AR expression (Antonarakis et al., 2014; Joseph et al., 2013). Thus, even at this stage, AR is still a potential drug target.

2.3.1 Therapies of local prostate cancer

About 80–85% of PCa cases are diagnosed as localized cancer and the remaining cancers are found in the advanced or the metastatic stage (Cooperberg et al., 2004; Li et al., 2018). For men diagnosed with localized PCa, the main treatment options include active surveillance, surgery, and radiation therapy (Table 1). Localized PCa can be curatively treated by surgical resection or by radiation therapy.

Active surveillance, with monitoring serum PSA, repeated prostate biopsies and/or MRI, is an accepted option for those low-risk patients who have local slow growing tumors that do not need aggressive immediate surgery or radiation therapy treatments (Lawrentschuk & Klotz, 2011; Parker et al., 2015). Active surveillance decreases overtreatments and maintains a good quality of life without the side effects of radical treatments, such as urinary and sexual dysfunctions (Punnen et al., 2013). However, patients receiving active monitoring had a higher risk of developing metastases than patients treated with surgery or radiotherapy (Hamdy et al., 2016). The overall 5-year, 10-year, and 15-year treatment-free rates in reported studies are 60% to 81%, 60% to 64%, and 43% to 55%, respectively (Klotz et al., 2015; Newcomb et al., 2016; Selvadurai et al., 2013; Tosoian et al., 2015; Welty et al., 2015).

The goal of radical surgery is to completely remove the localized prostate carcinoma tissue, and therefore, it requires careful preoperative and perioperative assessments of possible cancer invasion. Radical prostatectomy (RP) is usually

considered if the patient has a life expectancy of at least ten years, and the patient is aware of an increased risk of incontinence at an older age (Mottet et al., 2017). After radical surgery, serum PSA levels are expected to fall to barely undetectable levels within 2-6 months (Skove et al., 2017; Vesely et al., 2013), if the surgery has been curative. Subsequent increases in PSA values are almost invariably a sign of disease recurrence (Hong et al., 2010). Biochemical failure leads distant metastasis by about eight years (Pound et al., 1999).

Radiation therapy (RT) can be used as primary treatment or as an additional therapy after prostatectomy in men with locally advanced disease (Mottet et al., 2017). The RT was earlier used alone (Bagshaw et al., 1965), but it is nowadays used in combination with ADT to improve survival (Bolla et al., 2010; Pilepich et al., 2005). The combination with the ADT sensitizes PCa cells and increases their vulnerability to radiation damage by inhibiting the repair of DNA double-strand breaks, reducing hypoxic cells that would otherwise be radioresistant and metastatic, and increasing cell apoptosis and reducing mitoses (Locke et al., 2015). RT can be delivered as external beam therapy for locally advanced high-grade disease or localized brachytherapy, based on the implantation of radioactive seeds, for patients with low-to intermediate-risk local disease (Zaorsky et al., 2017). The most common adverse effects of RT are bowel dysfunctions (Donovan et al., 2016).

However, 20% to 40% of the patients who undergo radical prostatectomy (Freedland et al., 2005; Hull et al., 2002; Roehl et al., 2004), and 30% to 50% of the patients who undergo for radiotherapy (Kupelian et al., 2006), will have disease recurrence within ten years.

2.3.2 Therapies of local recurrence and metastatic prostate cancer

For advanced PCa, the first-line treatment is based on ADT (Cornford et al., 2017). The ADT by chemical or surgical castration, has been the main treatment of metastatic PCa since the 1940s (Huggins & Hodges, 1941). In addition, ADT is palliative in metastatic disease, it can be used as an adjunct therapy to RT and salvage treatment after RP or RT, but it is especially used as the first-line therapy in patients with metastatic disease at the time of diagnosis (Table 1).

The ADT prolongs OS, has an effect on metastases, relieves bone pain and decreases PSA levels in about 80–90% of metastatic diseases (Harris et al., 2009). Approximately 15% of the patients with metastatic PCa, fail to respond to ADT (Varenhorst et al., 2016). The duration of response to ADT varies widely, but approximately 5–10% of the patients remain alive 10 years after initiating treatment (Tangen et al., 2003). The adverse effects of ADT include e.g., fatigue, sexual dysfunction, hot flushes, anemia, neurophysiologic effects like the decreased mood

and cognition, osteoporosis which can lead to fractures, increased fat mass leading to insulin resistance and incident diabetes and increased cardiovascular risk factors (Grossmann & Zajac, 2011).

Standard treatment options for ADT are surgical or chemical castration with gonadotropin-releasing hormone (GnRH) agonist, such as goserelin and leuprolide, or antagonist degarelix (Cornford et al., 2017). These treatments reduce the secretion of luteinizing hormone (LH), and thus, prevent the formation of T in testicular Leydig cells (Figure 5) and in time ultimately results in down-regulation of GnRH receptors (Tolkach et al., 2013). It should be mentioned that chemical castration has also been found to have a reducing effect on adrenal androgen production (Miyazawa et al., 2017; Nishii et al., 2012).

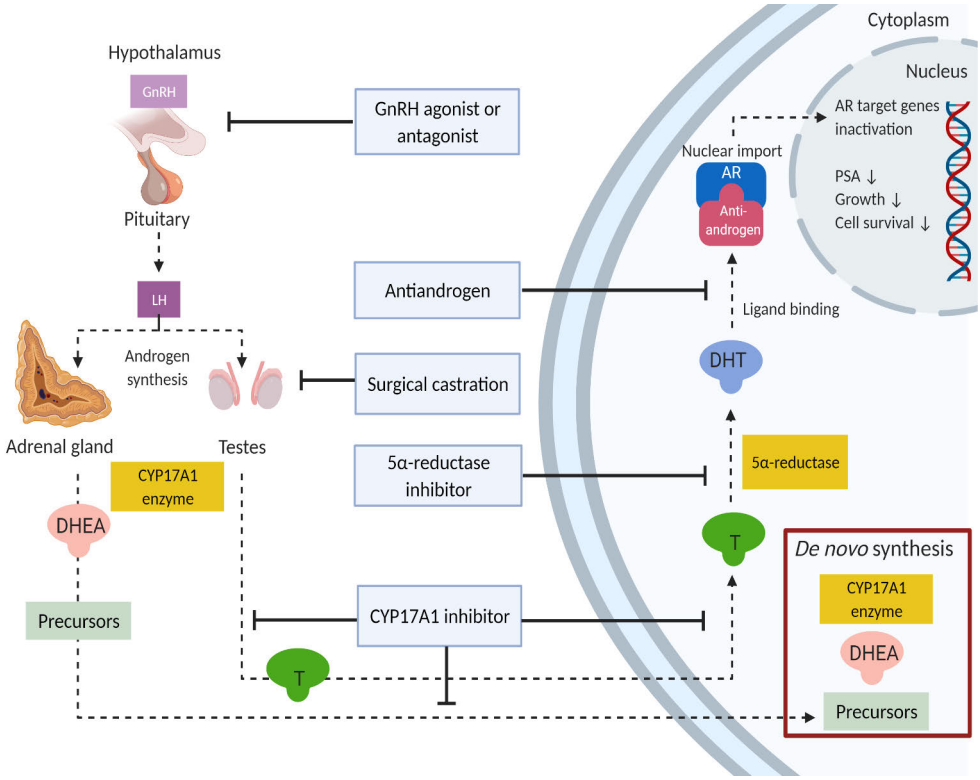

Figure 5. Mechanism of androgen receptor targeted therapies. Standard treatment options for ADT are surgical or chemical castration with gonadotropin-releasing hormone (GnRH) agonist or antagonist. These treatments act via GnRH receptors on the surface of gonadotropin cells in the pituitary gland, reduce the secretion of luteinizing hormone (LH), and thus, prevent the formation of testosterone (T). Cyp17A1 inhibitor, prevents not only testicular, but also adrenal and intratumoral androgen synthesis. Inhibitors of 5α-reductase prevent the conversion of T to the more potent androgen DHT, but although 5α-reductase inhibitors are effective in reducing symptoms associated with benign prostatic hyperplasia, their effect on prostate cancer remains controversial. Antiandrogen binding to AR prevents its activation. Created with BioRender.com

Surgical castration, orchiectomy (ORX), is a moderately simple procedure with minor surgical risks and it causes T levels rapid drop to the castrate levels (Desmond et al., 1988). Despite being an easy and a simple procedure, due to its negative psychological implications, it has been replaced with alternative medical options (McLeod, 2003). The efficacy of medical vs. surgical castration in advanced PCa has been compared in clinical trials with the results showing that survival, progression-related outcomes, and time for treatment failure is equivalent between these treatments (Seidenfeld et al., 2000). ORX remains still the treatment option for patients with spinal cord compression due to rapid effectiveness and pain relief for bone metastases, as the castration level is reached between 3 to 12 hours postoperatively (Rud et al., 2012).

For patients who no longer have the castration effect, medical or surgical castration can be combined with an antiandrogen to maximize AR activity or to prevent a tumor flare effect (temporary increase in testosterone levels) after initiation of a GnRH agonist. Older first-generation antiandrogens, flutamide and nilutamide, improved the survival of patients with metastatic PCa. However, the benefit was modest and should be balanced with increased adverse effects (Samson et al., 2002). Antiandrogen bicalutamide, with a better tolerability profile than flutamide and nilutamide, have shown more significant OS benefit and a better tolerability profile (Akaza et al., 2009).

In the prostate, the AR is expressed in both the epithelial and stromal cells (Prins et al., 1991; Singh et al., 2014). Androgen ablation results in prostate involution, and the loss of secretory luminal epithelial cells via apoptosis, but the apoptosis is not initiated in basal stem and transit-amplifying cells (English et al., 1987). Different sensitivities to androgen are explained by the strong AR immunoreactivity in the secretory epithelial cells, whereas the majority of basal epithelial cells are AR negative in the intact rat prostate (Prins et al., 1991).

As a first-line hormonal therapy in advanced PCa, the goal of ADT is to lower T levels and thereby, to reduce the tumor size or inhibit its growth. Serum T levels corresponding successful to castration, is generally set to 50 ng/dl (500 pg/ml, 1,7 nmol/l) (Bubley et al., 1999). However, levels below 20 ng/dl (200 pg/ml, 0,7 nmol/l) are reached in most men after castration, and it has been suggested that castration levels should be redefined to reflect this threshold (Crawford et al., 2011; Oefelein et al., 2000). T levels decrease to castration levels usually within 2–4 weeks (Klotz et al., 2008).

Although castration reduces circulating T level by 90–95% (Labrie, Dupont, & Belanger, 1985; Labrie et al., 1980; Moghissi et al., 1984; Waxman et al., 1983), a much smaller effect is seen intra-tissue androgen concentrations. The intra-prostatic DHT levels are reduced by only 50–70% following medical or surgical castration (Belanger et al., 1986; Labrie, Dupont, & Belanger, 1985). There are two different

forms of advanced and metastasized PCas: metastatic castration-resistant prostate cancer (mCRPC) and metastatic hormonal-sensitive prostate cancer (mHSPC). Although most patients with local recurrence and metastatic hormone sensitive PCa initially receive relief from ADT, the duration of response varies, and all develop incurable CRPC. The newer therapies have shown a marked survival advantage when added to ADT in the treatment of men with newly diagnosed mHSPC. Combining new therapies with ADT, such as chemotherapeutics or the androgen receptor targeted therapies, has emerged as a strategy to delay the development of CRPC and to improve the patient's quality of life and OS (Fizazi et al., 2017; Kyriakopoulos et al., 2018; Pang et al., 2019; Sweeney et al., 2015).

2.3.3 Therapies of castration-resistant prostate cancer

Despite the effective ADT, almost all patients will eventually progress to CRPC. Prior to 2010, taxane based chemotherapeutic agent, docetaxel, had been the first and only life-prolonging agent in mCRPC (Tannock et al., 2004). Many new CRPC therapeutics have been discovered over the last decade, e.g., CYP17A1 inhibitor abiraterone, the AR antagonist enzalutamide, the taxane cabazitaxel, the alpha-emitter radium-223, radiotherapeutical ^{177}Lu-PSMA-617, targeted therapy PARPi and the immunotherapy sipuleucel-T, have shown efficacy improving OS. Currently many novel agents such as AR targeting treatments, immunotherapeutics, oncogenic and genomic pathway targeted therapies are under clinical trials. However, despite the significant advances, CRPC remains a lethal disease but the early use and combination treatments of these novel agents, will likely in the future benefit the patient's management of the disease and quality of life. About 90% of CRPC patients develop painful bone PCa metastasis, and therefore, part of the therapy at this stage targets the bone health (Lukovic & Rodrigues, 2015). Metastatic PCa can cause severe bone pain most commonly in the vertebrae, pelvis, hips, or ribs (Suzman et al., 2014).

Approved drugs, other than hormonal drugs, have proven their place in the treatment of advanced PCa. For example, sipuleucel-T (currently available only in the US and withdrawn from Europe at the manufacturer's request) is an autologous cellular immunotherapy for the treatment of asymptomatic or mild symptomatic CRPC patients (Kantoff et al., 2010)**.** Today, chemotherapeutics docetacel and newer cabazitaxel are well-established treatment options that improves survival for patients with mCRPC (de Bono et al., 2010). The alpha-emitting bone target agent Radium-223, demonstrated a prolongation of OS compared to placebo after docetaxel settings and was approved for symptomatic bone mCRPC patients (Parker et al., 2013). The ^{177}-Lu-PSMA-617, specific drug that targets prostate-specific membrane antigen (PSMA) overexpressed in the PCa, has proved efficacy in clinical trials (Hennrich & Eder, 2022) (Fallah et al., 2023; Hennrich & Eder, 2022) and recently, PARP

inhibitors have also led to significant improvement in patients with certain genetic mutations (Taylor et al., 2023) . Table 2 lists additional drugs that act through different mechanisms for different stages of PCa.

Abiraterone**,** an androgen biosynthesis inhibitor, prevents not only testicular, but also adrenal and intratumoral androgen synthesis. It inhibits CYP17A1 enzyme that metabolize steroids such as pregnenolone and P_4 that provides intermediates for T and DHT synthesis. It is approved by the authorities for the treatment of mCRPC before and after the docetaxel chemotherapy (de Bono et al., 2011; Ryan et al., 2013) and later for the mHSPC patients (Fizazi, Tran, Fein, et al., 2017). Abiraterone decreases intratumoral DHT levels (Attard, Reid, et al., 2009), but it prolongs survival by only about 4 months in mCRPC patients who have received chemotherapy or without previous chemotherapy (De Bono et al., 2011; Ryan et al., 2013). In patients with newly diagnosed, metastatic high-risk HSPC and who had not received chemotherapy, abiraterone combination with prednisone reduced the risk of death by 38 percent compared to placebos (median OS not estimable/reached vs. 34.7 month)(Fizazi, Tran, Fein, et al., 2017). Adverse events of abiraterone are related to mineralocorticoid excess and hepatic injury (Ryan et al., 2015). Prednisone with abiraterone is co-administered to prevent hypertension, hypokalemia and fluid retention due to an excess of adrenocorticotropic-induced mineralocorticoids.

Enzalutamide is a second-generation AR antagonist and unlike their first-generation counterparts (e.g., bicalutamide and flutamide), it is more potent and displaces the DHT (endogenous ligand of the AR in the prostate gland) at lower concentrations than previous anti-androgens without any agonistic effects (Chen et al., 2004; Tran et al., 2009). In addition to being an active competitor for DHT, it reduces the binding of the AR to DNA, and inhibits the AR coactivator recruitment and therefore induces apoptosis (Scher et al., 2010). It is approved by the authorities for treatment of mCRPC (Scher et al., 2012), non-metastatic CRPC (nmCRPC) (Hussain et al., 2018) and mHSPC (Armstrong, Szmulewitz, et al., 2019).

More potent antiandrogens, called second-generation antiandrogens, have been designed to overcome castration resistance with maximum androgen blockade. These antiandrogens include enzalutamide (Xtandi), apalutamide (Erleada), and darolutamide (Nubeqa). Structurally related enzalutamide and apalutamide have 5 to 8 times higher binding affinity for AR compared to first-generation antiandrogens (Tran et al., 2009). The structurally different darolutamide shows 8- to 10-fold higher affinity for the AR than enzalutamide and apalutamide and can inhibit the clinically relevant AR mutations associated with enzalutamide resistance: AR F877L, W742L and T878A (previously reported in the literature as T877A) (Moilanen et al., 2015). Therefore, darolutamide is an even more potent second-generation antiandrogen.

Second-generation antiandrogens have succeeded in prolonging the survival of men with castration-resistant PCa. For instance, enzalutamide produced a 4.8-month

OS benefit after chemotherapy, compared to the placebo in men with mCRPC (Scher et al., 2012) and it also significantly decreased the risk of radiographic progression and death and delayed the initiation of chemotherapy in men with mCRPC (Beer et al., 2014). In addition, these second-generation antiandrogens have significantly prolonged metastasis-free survival in men with high-risk nmCRPC (Fizazi et al., 2019; Hussain et al., 2018; Smith et al., 2018).

Table 2. FDA approved treatment modalities of benign prostatic hyperplasia and PCa. Abbreviations: benign prostatic hyperplasia (BPH); PAP (prostatic acid phosphatase); mCRPC (metastatic castration-resistant prostate cancer); mHSPC (metastatic hormonal-sensitive prostate cancer); nmCRPC (nonmetastatic castration-resistant prostate cancer); prostate-specific membrane antigen (PSMA), PARP (poly ADP-ribose polymerase), skeletal-related events (SREs).

DRUG	TARGET	MECHANISM OF ACTION	INDICATION	REFERENCE
AR-TARGETED THERAPIES				
Abiraterone acetate	CYP17A1 inhibitor	Inhibits androgen synthesis in adrenal gland, testes and PCa cells	Approved to mCRPC and mHSPC	(de Bono et al., 2011), (Fizazi, Tran, Fein, et al., 2017)
Apalutamide	AR antagonist	Inhibits AR, thereby reducing the stimulation of PCa cells	Approved to mHSPC and nmCRPC	(May & Glode, 2019)
Bicalutamide, Flutamide, Nilutamide	AR antagonist		Approved combination with ADT	(Akaza et al., 2009), (Labrie et al., 1985), (Namer et al., 1988)
Darolutamide	AR antagonist		Approved to nmCRPC and mHSPC	(Fizazi et al., 2019), (Smith et al., 2022)
Degarelix acetate	GnRH antagonist	Inhibits LH release from the testes by binding to pituitary GnRH receptors decreasing circulating levels of T	Approved as ADT to HSPC	(Klotz et al., 2008)
Dutasteride	5α-reductase inhibitor	Block the conversion of T to the more active DHT	Approved to symptomatic BPH	(Roehrborn et al., 2002)
Enzalutamide	AR antagonist		Approved to nmCRPC, mHSPC and mCRPC	(Scher et al., 2010)
Finasteride	5α-reductase inhibitor		Approved to symptomatic BPH	(Gormley et al., 1992)
Goserelin, Histrelin, Leuprolide, Triptorelin pamoate	GnRH agonist	Cause an initial stimulation of the GnRH receptor, leading to a surge in T levels and later inhibit production of LH and thereby T	Approved as ADT to HSPC	(Turkes et al., 1987), (Chertin et al., 2000), (Herbst et al., 1984), (Parmar et al., 1987)

DRUG	TARGET	MECHANISM OF ACTION	INDICATION	REFERENCE
Chemotherapies				
Cabazitaxel	Mitotic inhibitor	Microtubule stabilization (anti-tubulin), interrupts cell cycle	Approved to mCRPC	(de Bono et al., 2010)
Docetaxel	Mitotic inhibitor	Anti-tubulin, AR signaling disruption	Approved to mCRPC	(Tannock et al., 2004)
Mitoxantrone	Type II topoisomerase inhibitor	Disrupts DNA synthesis and DNA repair	Approved to mCRPC	(Tannock et al., 1996)
Immunotherapies				
Dostarlimab	PD-L1 inhibitor, immunomodulator	IgG4-antibody (Anti PD-1), for all mismatch repair (MMR) deficient tumors	Approved to late stage and to certain mutations	(Andre et al., 2021)
Pembrolizumab	PD-L1 inhibitor	IgG4-antibody (Anti PD-1), for all mismatch repair (MMR) deficient tumors	Approved to late stage and to certain mutations	(Abida et al., 2019)
Sipuleucel-T	Autologous cellular immunotherapy, immunostimulant, vaccine	Immune cells of the patient have been stimulated to target the PAP	Approved to mCRPC	(Kantoff et al., 2010)
Bone-targeted therapies and radiotherapeuticals				
Bisphosphonates	Osteoclast inhibitor	Decreases bone resorption, prevention of skeletal-related events	Approved to prevention of SREs in mCRPC with bone metastases	(Macherey et al., 2017)
Denosumab	Human monoclonal antibody against RANK ligand	Decreases bone resorption by inhibiting osteoclasts and prevents skeletal-related events	Approved to prevention of SREs in mCRPC with bone metastases	(Smith et al., 2012)
Radium-223	Alpha-particle emitting radionuclide	Emits very low levels of radiation in bone causing double strand breaks in tumor DNA	Approved to mCRPC with bone lesions	(Parker et al., 2013)
^{177}Lu-PSMA-617	PSMA radioligand therapy	Specific drug that targets PSMA overexpressed in the PCa	Approved to mCRPC	(Hennrich & Eder, 2022)
PARP inhibitors				
Niraparib, Olaparib, Rucaparib	PARP inhibitor	Inhibit cancer cells to repair their damaged DNA leading to the death of the cells	mCRPC	(Sandhu et al., 2013) (Smith et al., 2019), (Mateo et al., 2015), (Fizazi et al., 2023)

2.4 Castration-resistant prostate cancer and resistance to second-generation hormonal therapies

Androgen deprivation therapy (ADT) with medical or surgical castration is the basis of PCa treatment. However, patients with advanced PCa who receive ADT almost consistently develop castration-resistant disease (Sharifi et al., 2005). CRPC is associated with a poor survival rate. About 10–20% of PCa patients develop castration resistance within approximately 5 years from diagnosis (Kirby et al., 2011). The median survival, since development of resistance, is about 14 months (in range of 9-30 months) depending on the patient's disease burden (Kirby et al., 2011). It is still not fully understood why PCa progresses to castration resistance, although mechanisms of resistance have already been extensively studied and many new therapeutic approaches have been developed. Progression to CRPC is typically diagnosed and defined with rising PSA despite castrate concentrations of T (< 1.7 nmol/l) (Cornford et al., 2017), suggesting pivotal AR-axis signaling (Ryan & Tindall, 2011; Scher & Sawyers, 2005). Earlier, CRPC was thought to be androgen independent. However, CRPC can grow in the presence of much lower levels of androgens than castration-sensitive PCas and androgens (T and DHT) remain high enough to activate AR in patients who have undergone chemical or physical castration (Mohler et al., 2004; Titus et al., 2005). With enzalutamide and abiraterone treatments, CRPC has been clinically proven to remain androgen-dependent both before and after docetaxel chemotherapy (de Bono et al., 2011; Ryan et al., 2013; Scher et al., 2012).

Enzalutamide is more potent than previous anti-androgens and displaces DHT at lower concentrations than previous anti-androgen bicalutamide (Tran et al., 2009) and the clinical studies have demonstrated the advantages of enzalutamide treatments. However, studies have also revealed that 22–46% of patients with CRPC did not respond to treatment with enzalutamide and the remaining 54–78% of enzalutamide-treated patients responded initially, but PSA progression could be observed after a median time of 8.3 and 11.2 months (Beer et al., 2014; Scher et al., 2012).

Abiraterone acetate effectively inhibits the enzyme CYP17A1, which is pivotal in the production of androgens. However, only 62% of chemotherapy naïve patients (Ryan et al., 2013) and 29,5% patients after chemotherapy (Fizazi et al., 2012) had PSA response (PSA decline ≥50% from baseline) and PSA progression could be observed after a median time of 11.1 (Ryan et al., 2013) and 8.5 months (Fizazi et al., 2012), respectively. Development of enzalutamide and abiraterone resistance are not fully elucidated, but several molecular mechanisms leading to resistance are similar to the mechanisms of CRPC, including androgen receptor-related signaling

pathways, glucocorticoid receptor-related pathways and steroid synthesis related mechanisms explained more below.

Despite the improved response rates and OS of patients with abiraterone and enzalutamide treatment, patients either have primary resistance or more likely they develop resistance to these agents during treatment. Treatment resistance to the targeted therapies such as antiandrogens, mainly arises via cellular rewiring by enabling alternative bypass signaling pathways after therapy exposure and, therefore, allowing continued tumor proliferation and survival (Carceles-Cordon et al., 2020). Resistance can also be explained by the clonal evolution model, in which genomic instability enables the existence of intratumoral heterogeneity and explains the presence of distinct subgroups of cancer cells at the tumor molecular level (Beltran et al., 2016). Adaptive resistance to second-generation hormonal therapies e.g., enzalutamide treatment, can be due to the activation of both AR-dependent pathways and mechanisms not dependent on AR signaling pathways.

2.4.1 AR related mechanisms

Despite very low circulating levels of serum T, the AR signaling is maintained by multiple mechanisms including activating AR mutations or truncations, AR amplification or overexpression resulting in increased protein expression, changes in AR cofactor balance and extragonadal androgen production, including in the tumor tissue itself. Therefore, many of the AR-regulated target genes that promote the growth and survival of PCa are still expressed in the tumor cell. One of the major goals of the PCa research is to identify these key AR-regulated genes and to further investigate them as therapeutic targets for the treatment of advanced PCa.

It is known that therapies leading to suppression of androgen action may lead to the development of different AR pathway related resistance mechanisms listed below.

- The induction of intratumoral androgen (T and DHT) biosynthesis *de novo* or from circulating precursors derived from the adrenal gland (Cai et al., 2011; Locke et al., 2008).
- Overexpression of AR due to e.g., AR gene amplification or increased transcription (Chen et al., 2004; Koivisto et al., 1997; Linja et al., 2001).
- Somatic point mutations in the AR gene, causing a change of the AR ligand binding pocket so that other steroids, corticosteroids or antiandrogens can activate the mutated forms of AR (Bohl et al., 2005; Korpal et al., 2013; Sun et al., 2006). E.g., mutation F876L converts enzalutamide and apalutamide from AR antagonists into AR agonists *in*

vitro, with several cases documented clinically (Balbas et al., 2013; Joseph et al., 2013).

- Alternative AR gene splicing, producing ligand-independent and/or constitutively active AR splice variants (Antonarakis et al., 2014, 2017; Sprenger & Plymate, 2014; Sun et al., 2010; Watson et al., 2010).
- Upregulation of the glucocorticoid receptor (GR) or progesterone receptor (PR) in tumor cells are also suggested to provide a mechanism to bypass AR blockade. In this case AR target genes are regulated via an alternative nuclear receptor (Arora et al., 2013; Puhr et al., 2018)
- Altered expression and function of AR co-regulators. AR co-regulators are proteins e.g., enzymes that assistant the activation of AR transcription and have a role in its activation (coactivators) or inhibition (corepressors) of AR-mediated transcription (Heinlein & Chang, 2002).
- Aberrant post-translational modifications of AR (Coffey & Robson, 2012; Culig, 2016; Samaržija, 2021; Wolf et al., 2008). Post-translational modifications of AR mainly include acetylation, methylation, ubiquitination, sumoylation and phosphorylation contributing to the regulation of AR structure, activity and stability.

2.4.1.1 Androgen biosynthesis upregulation

PCa cells can upregulate steroidogenesis within prostate tumors thus enabling them to synthesize endogenous androgens for their own use and survive with ADT by converting androgen precursors derived from adrenal glands or by *de novo* synthesis from cholesterol (Montgomery et al., 2008; Stanbrough et al., 2006).

In patients treated with abiraterone, it is thought that the accumulation of steroids upstream of CYP17A1, such as 5α-progesterone and allopregnanolone, may contribute to the steroids production of an alternative route (Attard et al., 2008). These findings suggest that the classical T-biosynthetic pathway alone is no longer sufficient to describe human androgen biology as hormonal changes. Due to illness or some other causes, the body cause adaptation in the androgen synthesis and the use of alternative pathways.

The alternative backdoor pathway for the DHT biosynthesis, bypassing A-dione and T production, was first demonstrated in the tammar wallaby pounch young testis (Shaw et al., 2000; Wilson et al., 2003). Since then, alternative DHT synthesis pathways have been found to contribute intratumoral DHT synthesis via bypassing T production (Chang et al., 2011; Penning, 2014), DHT production directly from A-dione and androstanedione (5α-dione) via reductase activity (SRD5A1), has been described in CRPC cell lines and human tumor metastases (Chang et al., 2011) and

a similar steroid profile has also been found in polycystic ovary syndrome patients (Fassnacht et al., 2003). The 5α-dione pathway would appear to be the preferred pathway for adrenal androgen precursors in many PCa cell lines as well as in metastatic CRPC biopsies taken from patients (Chang et al., 2011). This could be explained with the upregulated expression of 5α-reductase SRD5A1 isoenzymes in high-grade and CRPC tumors (Thomas et al., 2008) and that A-dione is a better substrate for this enzyme than T (Chang et al., 2011). Also, another alternative backdoor pathway key enzymes, AKR1C3 is found to be overexpressed in both abiraterone- and enzalutamide-resistant PCa cells (Liu et al., 2015, 2017).

Clinically, it has been observed that enzalutamide increased circulating DHT, T and A-dione levels prior to prostatectomy (Montgomery et al., 2017). This finding is consistent with previous studies showing that antiandrogens eliminate negative feedback from the hypothalamus and thus increase androgens and estradiol (Eri et al., 1995). The additive effects of enzalutamide on prostate or circulating androgen levels in the castrate setting are unknown.

2.4.1.2 AR gene amplification and overexpression

AR gene amplification leading to AR protein overexpression is associated with increased cell proliferation. Amplified copy number and increased expression of AR surges AR response to low androgens levels (Visakorpi et al., 1995). It is the most common genetic change among CRPC patients treated with ADT, with up to 80% of reported to carry an elevated AR gene copy number and approximately 30% has a high-level gene amplification (Koivisto et al., 1997; Taylor et al., 2010; Visakorpi et al., 1995).

In CRPC patients treated with abiraterone and enzalutamide, AR overexpression was observed in 45% of patients during the treatment and overexpression was more common in patients treated with enzalutamide than abiraterone (53% vs. 17%) (Azad et al., 2015). Additional studies of liquid biopsies have also demonstrated that AR amplification in circulating tumor DNA (ctDNA) is associated with resistance to abiraterone and enzalutamide (Wyatt et al., 2016). In a study using ctDNA for examining the genomic landscape of the AR, indicated that the patients with AR amplification had worse prognosis. In this study, 50% of patients pretreated with either enzalutamide or orteronel (an inhibitor of CYP17A1) prior to abiraterone treatment showed AR amplification, and only 13% of those had a response of ≥ 50% of PSA decline after being treated with abiraterone (Romanel et al., 2015).

2.4.1.3 AR mutations

Structural changes in the AR, induced by mutations and aberrant transcription, are rare in the early stages of PCa, but it is commonly present in CRPC, after hormone therapies. In CRPC, AR mutations are found in 5–30% of tumors, (Azad et al., 2015; Romanel et al., 2015) and in approximately 12% to 48% of CRPC patients receiving enzalutamide or abiraterone (Waltering et al., 2012; Wyatt et al., 2016). There are more than a hundred point mutations in an AR gene and most of them occur in the NTD or LBD regions, but the majority of clinically-relevant somatic point mutations in the AR are located in the LBD (Azad et al., 2015; Hara et al., 2003; Taplin et al., 1995). Mutations in AR commonly lead to increased receptor transactivation activity and decreased ligand specificity meaning AR activation by alternative ligands such as adrenal androgens, other steroid hormones and AR antagonists.

One of the common and most investigated forms of AR mutations is the T878A (previously reported in the literature as T877A) substitution. It occurs in patients after prolonged ADT or abiraterone treatment leading to an expansion of the ligand binding specificity of AR that allows the ligand binding of other steroid hormones such as P_4 and estrogen (Chen et al., 2015; Steketee et al., 2002). Furthermore, this mutation allows the activation of AR in addition to P_4 by other adrenal androgens such as DHEA (Tan et al., 1997) and androstenediol (Miyamoto et al., 1998). It should also be noted that as serum P_4 increases during abiraterone therapy and therefore this mutation may play a role in abiraterone resistance (McKay et al., 2017).

Several mutations can turn antiandrogenic agents into agonists. For example, the F877L mutation activated by enzalutamide or apalutamide (former ARN-506) has been shown to convert AR antagonists to AR agonists in a PCa cell line after long-term treatment (Balbas et al., 2013; Joseph et al., 2013). Other mutations, like L702H, may confer agonism with glucocorticoids (Lallous et al., 2016).

2.4.1.4 AR splice variants

AR splice variants (AR-Vs) are truncated forms of AR lacking the androgen receptor ligand-binding domain (LBD). These truncated versions of AR are often constitutively active because they do not require a ligand to initiate downstream AR signaling. Alternative AR gene splicing has been shown to be associated with resistance to ADT as well as the resistance to abiraterone and enzalutamide (Nakazawa et al., 2014).

More than 20 AR variants have already been identified, but only some variants such as AR-V7, AR-V1, AR-V3, AR-V9, and ARv567es have been studied in more detail (Armstrong & Gao, 2019). Many studies have shown AR-V7 to be the most abundantly expressed variant. AR-V7 protein expression is rare in the primary

tumors (<1%), but more common in CRPC tumors (>75%) (Sharp et al., 2019). Resistance to both enzalutamide and abiraterone has been found to increase AR-V7 expression levels, and its expression in patients correlates significantly with a lower PSA response and shorter progression-free and OS compared to men without AR-V7 expression (Antonarakis et al., 2014). Although AR-V7 alone does not drive PCa cell line proliferation when full length AR is absent (Kregel et al., 2020; Watson et al., 2010) many studies support its role for development of treatment resistance.

A number of the AR-Vs (including AR-V7) in patients with CRPC, primarily localize in the nucleus, while others (including AR-V1, AR-V4 and AR-V6) localize mainly in the cytoplasm and frequently co-expressed with the nucleus-predominant AR-Vs as well as the full-length AR (AR-FL) (Zhan et al., 2017). This suggests that different AR-Vs have co-operation with each other and with full-length AR (Kallio et al., 2018; Zhan et al., 2017).

The functional roles of the AR variants are not yet fully understood partly due to the lack of accurate (Armstrong & Gao, 2019) AR variant-specific antibodies. However, there are many studies ongoing targeting the AR variants to improve treatment response in AR related therapy resistant PCas with a high prevalence of AR variant expression. Many new compounds and different strategies have been investigated to the inhibition of full-length AR and variants activity or expression. These are e.g., AR variant degradation (Liu et al., 2014, 2016), targeting the N-terminal domain (Yang et al. 2016) and DNA binding domain by antagonists (Dalal et al., 2014), inhibition of AR variant synthesis (Ferraldeschi et al., 2016; Wang et al., 2017) and inhibition of AR variant co-activators (Magani et al., 2017).

2.4.1.5 AR-interacting coregulators and post-translational modifications

AR coregulators are protein factors involved in the activation of AR transcription and play a role in the activation or inhibition of AR-mediated transcription. More than 280 proteins have been isolated as AR-interacting coregulators that can affect either positively (coactivators) or negatively (corepressors) transcription of AR target genes (Depriest et al., 2016; Heemers & Tindall, 2007). The AR-associated coregulators have general a high diversity of biological utilities and pathways that promote the regulation of AR function (Heemers & Tindall, 2007) such as activating the transcriptional function of AR at very low androgen levels (Hermanson et al., 2002).

Androgens have been shown to increase and stabilize AR protein levels in various cell contexts (Lee & Chang, 2003; Yeap, 1999). The half-life of AR protein varies depending on intracellular factors, but in addition to androgen regulation, AR is also regulated by post-translational modifications (Coffey & Robson, 2012). Many of AR coregulators are enzymes that modulate other proteins through

phosphorylation, acetylation, methylation, ubiquitination, and SUMOylation (Coffey & Robson, 2012; Culig, 2016; Wolf et al., 2008). These modifications activate or suppress the transcriptional activity of AR, leading to the regulation of AR protein stability, intracellular localization, transcriptional activity and expression regulation of its target genes (Coffey & Robson, 2012; van der Steen et al., 2013). The stability of AR, i.e., a longer half-life of the protein, is one mechanism that may lead to treatment resistance to ADT through decreased degradation or through increased translation (Santer et al., 2015).

Phosphorylation is the most common post-translational modifications of AR, and most AR phosphorylation occurs in the presence of androgens, while some of them are androgen-independent (Wen et al., 2020). CRPC cells may continue to grow because coactivators, such as ACK1 and SRC, and their downstream tyrosine kinases, that are elevated during hormone ablation therapy, may induce tyrosine phosphorylation of AR leading to sensitization of AR to low hormone levels or even androgen-independent activation of AR (Guo et al., 2006; Mahajan et al., 2007).

2.4.2 Bypassing mechanisms of AR

2.4.2.1 Glucocorticoid and progesterone receptor

Glucocorticoids, normally secreted by adrenal glands, are used in synthetic forms e.g., in chemotherapy to reduce inflammation and cytotoxic side effects (Montgomery et al., 2014). In androgen-dependent PCa, glucocorticoids appear to slow the proliferation of tumor cells, whereas in CRPC, glucocorticoids act in different ways, leading to tumor progression (Montgomery et al., 2014, 2015). It has been shown that dexamethasone, that is a potent GR agonist, conferred resistance to enzalutamide, whereas GR antagonists restored sensitivity (Arora et al., 2013). However, inhibiting glucocorticoid signaling is neither practical nor effective because the glucocorticoid is essential for life and because GR antagonists can activate AR target genes (Arora et al., 2013; Klokk et al., 2007; Sharifi, 2014).

The glucocorticoid receptor (GR) belongs to the same nuclear steroid receptor class of AR sharing same response elements in target genes and therefore its activation can regulate AR target genes expression (Arora et al., 2013; Isikbay et al., 2014). GR expression is low in primary PCa tissue, and it occurs in approximately 30% of PCa cases, however, expression may be increased in patients following ADT and during long-term enzalutamide treatment leading treatment resistance (Arora et al., 2013; Puhr et al., 2018; Szmulewitz et al., 2012).

The level of active ligand for GR, cortisol, has also been observed being up-regulated after enzalutamide treatment by 11β-hydroxysteroid dehydrogenase-2 (11β-HSD2) inactivation (Li et al., 2017), which normally enzymatically converts

cortisol to inactive cortisone in humans and corticosterone to 11-dehydrocorticosterone in mice (Chapman et al., 2013). Decreased androgens and increased serum glucocorticoids may lead to the presence of AR variants with mutations in LBD, allowing their activation by glucocorticoids (Lorente et al., 2014) and therefore GR or other nuclear steroid receptors can bypass AR pathways and promote CRPC development.

Progesterone receptor (PR) and AR similarity is also detrimental in CRPC. Similarly, to GR, PR has the ability to transcriptionally regulate a subset of AR target genes in PCa and thus bypass AR (Chen et al., 2017). High levels of PR have also been found from tumor samples in clinical trials to be associated with cancer recurrence and to correlate with shorter OS (Grindstad et al., 2015) and thus, in preclinical studies with tumor models, inhibition of PR and GR activity by mifepristone, a steroidal GR and PR antagonist, inhibited CRPC growth and delayed progression (Isikbay et al., 2014).

2.4.2.2 AR-dependent fusion proteins

The gene fusion between TMPRSS2 (encoding a transmembrane serine protease constitutively expressed in prostate) and ETS (erythroblast transformation specific) family genes (ERG, FL11, ETV4 and ETV5) are most prevalent molecular alterations in PCa, occurring in 40–50% of tumors (Tomlins et al., 2005). These alterations can lead to ETS protein abnormal expression, abnormal activation of downstream target genes of ETS, abnormal cell proliferation (Tomlins et al., 2005) and eventually worse clinical prognosis (FitzGerald et al., 2008). Finding that ERG fusion-positive PCa patients responded better to abiraterone treatment compared to ERG fusion-negative patients (Attard, Swennenhuis, et al., 2009), lead to suggestion that ERG may regulate the synthesis of intratumoral androgens. Indeed, the AR regulated TMPRSS2-ERG fusion protein, which up-regulates AKR1C3 expression in advanced PCa, together forms ERG/AKR1C3/AR feed-forward loop that maintains androgen synthesis and promotes AR signaling and CRPC growth (Knuuttila, Mehmood, Mäki-Jouppila, et al., 2018; Powell et al., 2015). The TMPRSS2-ERG fusion has also been found to be associated with docetaxel resistance in mCRPC patients (Galletti et al., 2014).

2.4.3 AR independent mechanisms

As discussed above, most of the mechanisms responsible for disease progression in CRPC are related to the maintenance of AR signaling. However, also other pathways are essential in PCa growth. Studies in mCRPC patients have shown tumor accumulation that does not express AR after ADT treatments (Roudier et al., 2003;

Shah et al., 2004) and these tumors exhibit increased interaction between other molecular pathways and AR signaling (Pisano et al., 2021).

2.4.3.1 Neuroendocrine differentiation

During the initial diagnosis of PCa the neuroendocrine prostate cancer (NEPC) is rare and ranges from 0.5 to 2% of all PCa cases (Patel et al., 2019). These NEPCs are composed of neuroendocrine tumor cells and diagnosed outside the context of a previously known adenocarcinoma. However, studies have suggested that NEPC could also arise from the trans-differentiation of adenocarcinoma, CRPC cells or cancer stem cells (Patel et al., 2019). The NE phenotype is characterized by rapid disease progression, and a systematic review of published clinical cases revealed that the median time to development of NEPC is 20 months and the median OS after NEPC diagnosis is 7 months (Wang et al., 2014).

Although NEPC may arise *de novo*, most cases occur in CRPC patients treated with hormonal therapy and/or taxane-based chemotherapy. It has been recently recognized that the use of more effective hormonal therapies, such as enzalutamide, contributes to a 15–30% increase in the incidence of therapy-induced neuroendocrine prostate cancer (t-NEPC) (Aggarwal et al., 2018; Patel et al., 2019). The t-NEPC does not express, or expresses only moderate levels, of luminal prostate differentiation markers such as PSA, but they do express biomarkers such as chromogranin A (CHGA), neuron-specific enolase (NSE) and synaptophysin (SYP) (Beltran et al., 2016). Although some AR-negative tumors express markers of neuroendocrine differentiation, the diverse phenotype of these NEPCs ranges from anaplastic carcinomas to mixed prostate adenocarcinomas with neuroendocrine-like small cell carcinomas or to pure small-cell carcinomas (Aparicio et al., 2011; Beltran et al., 2011; Tzelepi et al., 2012).

There are several potential models of tumor progression toward the neuroendocrine phenotype, but the signaling mechanisms by which neuroendocrine differentiation occurs are not yet known, making it a challenge in the development of therapeutic interventions (Beltran et al., 2016; Patel et al., 2019).

2.4.3.2 Crosstalk with other signaling pathways

Although the role of AR in PCa is central, other signaling pathways and their interaction with AR signaling also play a significant role in the progression of PCa. Alternative pathways may facilitate the identification of new therapeutic targets for PCa as many cellular signaling pathways that are involved in cell growth, proliferation, and survival, including mTOR and Wnt pathways, are associated with CRPC (Kato et al., 2016).

Several studies have shown that regulation of the PI3K/AKT/mTOR pathways is associated with the progression of PCa due to tumor suppressor phosphatase and tensin homolog (PTEN) loss (Carver et al., 2011). Loss of PTEN function through deletion, mutation, or decreased expression occurs in an estimated 20% of primary prostate tumors and in 50% of CRPC tumors (Jamaspishvili et al., 2018). The PI3K/AKT/mTOR pathways, on the other hand, have been found to be altered in nearly 50% of primary PCas and 49 to 100% of mCRPCs (Robinson et al., 2015; Taylor et al., 2010). PTEN-deficient preclinical models have shown that the AR and PI3K/Akt pathways regulate each other with reciprocal feedback, which maintains tumor cell survival (Carver et al., 2011; Thomas et al., 2013), and promising results have been obtained in clinical studies combining enzalutamide and pan-AKT inhibitor in patients with mCRPC with PTEN loss (de Bono et al., 2019; Kolinsky et al., 2020).

The Wnt pathway is involved in cell proliferation, apoptosis, adhesion, migration, polarity, epithelial and mesenchymal transition (EMT), and transcriptional activation of some metastatic factors (Placencio et al., 2008). It has been widely reported in other cancers and activation of Wnt signaling has also been observed in CRPC (Beildeck et al., 2010; Wang et al., 2008). The Wnt signaling pathway is generally inactive in normal prostate cells but has been found to be altered in approximately 18% of mCRPC (Robinson et al., 2015). Patients with somatic Wnt-pathway activating mutations have been found to have worse outcomes to first-line abiraterone or enzalutamide treatment than Wnt wild-type patients (Isaacsson Velho et al., 2020). Thus, abiraterone and enzalutamide treatment in CRPC patients may activate the Wnt signaling pathway leading to androgen-independent PCa growth.

2.4.4 Chemoresistance

Cytotoxic drugs play an important role in prolonging the time to the progression of metastatic disease, alleviating symptoms, and improving OS. Taxanes, e.g., docetaxel and capazitaxel, stabilize microtubules and prevent separation of chromosomes, leading to mitotic arrest and consequent apoptosis (van Soest et al., 2015). The cytotoxic drug docetaxel was the first and only life-prolonging agent in mCRPC before 2010 (Petrylak et al., 2004; Tannock et al., 2004). However, resistance to docetaxel develops and the disease progresses in an average of 8 months (Tannock et al., 2004).

The development of PCa chemoresistance can develop through a variety of mechanisms, such as up-regulation of cell membrane efflux transporters and influx down-regulation, thereby decreasing the intracellular concentration of cytotoxic agents (Huang & Sadée, 2006). In addition, mutational changes in tubulin reduce the

binding of taxanes and thus prevent apoptosis (Hara et al., 2010) Long-term docetaxel treatment has also been found to increase the expression of pAKT and thus induce the up-regulation of the PI3K/AKT signaling (Crabb et al., 2017; Kosaka et al., 2011) .

It is known that one of the docetaxel mechanisms is that it interferes with AR signaling. Cross-resistance with docetaxel and enzalutamide was shown in an *in vivo* study with patient-derived enzalutamide-naïve and entzalutamide-resistant tumors. The study showed that docetaxel inhibited tumor growth, AR nuclear localization and AR-regulated gene expression in enzalutamide-naïve tumors but not in the enzalutamide-resistant tumors (van Soest et al., 2015). In contrast to docetaxel, in the same study, cabazitaxel remained highly effective in enzalutamide-resistant tumors and showed AR-independent antitumor activity compared to docetaxel.

2.5 Strategies to overcome the resistance

Today, we know that metastatic PCa is a heterogeneous disease in which AR-positive and AR-independent neoplastic cells co-exist and inhibition of AR-positive cells can cause treatment resistance by clonal selection, and the consequent growth of AR signaling-independent cell clones (Pisano et al., 2021; Roudier et al., 2003; Shah et al., 2004). Eight therapies (abiraterone, docetaxel, enzalutamide, radium-223, ^{177}Lu-PSMA-617, cabazitaxel, olaparib and sipuleucel-T) have been approved and recommended, to improve the OS of patients with mCRPC. Metastatic PCa patients, who initially benefit the treatments, will eventually progress to the refractory stage mainly due to altered signaling regulation leading to cell regrowth, proliferation, and survival.

The recently approved nmCRPC and mHSPC antiandrogen treatments with apalutamide and darolutamide are more potent than enzalutamide and show lower brain penetration and a lower risk of seizures (Fizazi et al., 2019; Smith et al., 2018). In addition, darolutamide is effective in blocking known AR mutations (Moilanen et al., 2015). Other promising therapies affecting AR are constantly being investigated. For example, patients who are resistant to abiraterone or anti-androgens with constitutive activated expression of AR-Vs, could achieve therapeutic responses from compounds that target the constitutively active ligand-independent transcriptional activation of the N-terminal domain of AR (Leung et al., 2021). Another approach is AR degraders, using PROteolysis TArgeting Chimeric (PROTAC) technology, which induces AR degradation through proteasomal degradation, resulting in more potent anti-proliferative, pro-apoptotic effects and attenuation of AR target gene expression in PCa cells (Kregel et al., 2020).

Treatments with entirely new mechanisms of action, such as immunotherapies and poly ADP-ribose polymerase (PARP) inhibitors (Lord & Ashworth, 2017;

Topalian et al., 2015) and prostate-specific membrane antigen (PSMA)-targeted therapies (Donin & Reiter, 2018), are also highly researched as potential strategies to treat this unmet need. In addition to new targeted drugs, several treatment strategies are currently being developed to delay treatment resistance, of which a few are discussed below.

One approach that has been shown to be effective in other cancers is to delay the development of resistance with combination therapies, where the combination of the two or three drugs can prevent or slow the onset of acquired resistance more effectively than either agent alone. This has been tested e.g., by co-targeting the AR and androgen synthesis inhibition with enzalutamide and abiraterone in mCRPC. Enzalutamide in combination with abiraterone had a manageable safety profile without significant drug interactions, but unfortunately, efficacy results did not support the significant benefit of this combination therapy in mCRPC (Efstathiou et al., 2020; Morris et al., 2019). Another example of combinations is the administration of chemotherapy with endocrine therapy to also disrupt AR-independent survival pathways and AR-negative cells. Such an approach may delay the development of therapeutic resistance, and clinical trials have shown that patients treated with multiple therapy combinations have significantly better OS than patients treated with ADT alone (Kyriakopoulos et al., 2018; Sweeney et al., 2015). One novel example is the combination of darolutamide with ADT and docetaxel, which together increased OS in patients with mHSPC without increasing adverse events (Hussain et al., 2023). A third possibility of combination treatments is, that after disease progression, enzalutamide or abiraterone is combined with another agent to target another resistance and signaling pathway, e.g., enzalutamide or abiraterone with an AKT inhibitor (de Bono et al., 2019; Kolinsky et al., 2020) or PARP inhibitor. Inhibition of PARP blocks cancer cell ability to fix DNA damages, which are normally repaired by the homologous recombination repair during the cell cycle, leading eventually to cancer cell death (Lord & Ashworth, 2017). Additionally, PARP inhibitors suppress AR target-gene expression, and thus, increase the effect of inhibition of tumor proliferation (Schiewer et al., 2012). Indeed, early clinical data is promising for the combination of PARP inhibitors with antiandrogens and immunotherapy in PCa (Nizialek & Antonarakis, 2020). A lot of progress has been made recently. The PARP inhibitor combination with abiraterone and prednisone or prednisolone is already approved in the EU and several other countries for the treatment of adult mCRPC patients (Clarke et al., 2022), and an FDA decision on the use of a combination of enzalutamide and a PARP inhibitor is expected in 2023 for the same indication (Agarwal et al., 2023).

PCa treatment typically consists of monotherapies prescribed in sequence. Another possible method is to use two different agents in an optimal order, with patients starting with one treatment and then switching to another drug. For example,

treatment with enzalutamide and abiraterone has been tested clinically whether either sequence is superior to the other with the result that abiraterone followed by enzalutamide provides the greater clinical benefit than vice versa in mCRPC (Khalaf et al., 2019; Mori et al., 2020).

One more example of an option is to use high-dose testosterone in patients with CRPC who have become resistant to ADT treatment. In this method, referred to as bipolar androgen therapy (BAT), approach is to re-challenge, e.g., enzalutamide resistant mCRPC, allowing the tumor cells to cope with from supraphysiological levels of testosterone to near-castrate levels by rapidly cycling between androgen stimulation and deprivation (Schweizer et al., 2015). Based on promising studies, mCRPC patients who progressed with enzalutamide showed re-sensitivity to the drug when patients were re-exposed to antiandrogens as testosterone treatment progressed (Teply et al., 2018). In addition to the fact that the treatments have been able to sensitize the cancer of CRPC patients to subsequent endocrine treatment, it has also helped to improve the patients' OS and their quality of life (You et al., 2023).

2.6 Animal models of prostate cancer

Animal models, especially mouse models, have a key role in studies modelling human PCa in its various phases. Although cell and tissue culture models are very useful in understanding the biology of PCa and early-phase drug testing, they lack complex cell interactions expressing tumor microenvironment that plays a vital role in cancer development and progression. In addition to the body's complex hormonal regulatory factors, blood vessels, immune cells, fibroblast, and the nervous system also interact with PCa cells, and the circulating oxygen and nutrients play an essential role in regulating tumor growth.

PCa occurs spontaneously in dogs and some rat strains (Rosol et al., 2003). Although dogs have many similarities to human PCa (LeRoy & Northrup, 2009), there are limitations to their use. PCa is rare in dogs and occurs in less than 1% of dogs (Bryan et al., 2007). In addition, dogs also have a relatively long disease latency and their high maintenance costs and difficulty in genetic manipulation make it an unrealistic experimental model. Also with rats, tumor rarity, phenotypic variability, long latencies, and the absence of metastases, limit their use in the PCa carcinogenesis studies, but instead chemically induced and genetically modified and implanted xenograft rat models are used to some extent to model PCa (Nascimento-Gonçalves et al., 2018).

The goal of animal disease models is to mimic human disease in order to discover the molecular mechanisms of the disease and to test new therapies. The ideal animal model should be simple, inexpensive, and mimic human disease as much as possible. PCa is usually a slow-growing cancer that occurs late in life, and therefore, modeling

these characters in mice and designing *in vivo* experiments is difficult. Therefore, no single animal model faithfully covers the entire spectrum of human PCa progression.

The use of mice as a research model for PCa, has made a significant contribution to the study of the progression and treatment modalities of PCa. Mouse models of PCa are most commonly either genetically modified (GM) mouse models or xenograft models. Significant advantages of GM models are that they reflect tumor progression over time from the onset of preinvasive lesions to invasive and even metastatic lesions in the prostate microenvironment, including the fully intact immune system of the body, and therefore, they are good models for elucidating molecular pathways in the development and progression of PCa (Kaplan-Lefko et al., 2003; Shukla et al., 2005). New-generation GM models using chemically activated Cre recombinase may allow faster induction of cancer in the model, and thus, cancer activation in the model is also possible at a later age. The disadvantage of using GM mice is the significant biological differences between mouse and human prostate (Ittmann, 2018). In addition, GM models are also more time-consuming and expensive compared to the most commonly used xenograft models. However, GM models can be superior when studying compounds that target a particular molecular pathway that is activated in this model by genetic manipulation.

Because one model does not cover the entire course of PCa, a variety models are needed to study the different stages of the disease. A common *in vivo* PCa model is the transplantation of human tumor tissue or cells (xenografts) into immunodeficient mice, by various techniques (i.e., subcutaneous, orthotopic, intravenous or intracardiac inoculations). The advantages of *s.c.* xenograft models include an easy inoculation technique and easy growth monitoring, and a large amount of tumor tissue obtained for further studies, but *s.c.* xenografts almost never metastasize. Orthotopic (*o.t.*) xenograft models, on the other hand, allow the study of the interaction between cancer and the target organ. Furthermore, orthotopically inoculated PCa cells proliferate not only in the prostate tissue, but they may also spread to the surrounding tissues and may metastasize to other organs, such as the lymph nodes. (An et al., 1998). Inoculated xenograft models are most used for drug treatment studies due to their reproducibility, quick experimental time frame and relative ease of experimental setup. Immunodeficient mice are used because they are unable to generate an immunological response to foreign tissue, allowing human tumors to grow in mice, and therefore, the role of the immune system cannot be studied in this model.

Established cell lines are commonly used in xenograft models because freshly isolated PCa cells and tissue fragments rarely form tumors in mice (Pienta et al., 2008). One of the problems with PCa modeling is the lack of multiple cell lines. Other cancers have dozens of cell lines, but the majority of PCa studies with cell lines are performed with PC3, LNCaP (and its derivatives), 22Rv1, VCaP, MDA and

DU145 cell lines (Pienta et al., 2008; Valkenburg & Pienta, 2015; Woods-Burnham et al., 2017). Human PCa has proven to be very difficult to grow in the laboratory either *in vitro* or *in vivo* as PCa is generally slow growing, morphologically, and molecularly heterogeneous, and highly dependent on paracrine and endocrine signals (Pienta et al., 2008). This has prevented the widespread use of patient derived xenograft (PDX) models in research, although it better mimics the condition and characteristics of patients and the treatment response to drugs. However, representative PCa PDX models such as LAPC, KUCaP and LuCaP cell lines are in use, although they are derived from patients with advanced-stage PC and represent only a small proportion of phenotypes (Corey et al., 2003; Klein et al., 1997; Terada et al., 2010).

The use of *s.c.* or intraprostatic xenograft models is one of the most reproducible ways to study human PCa *in vivo* and has yielded significant information about the course of the disease. One notable example of this is the modelling of PCa progression to castration-resistant disease after androgen ablation treatment. In the CRPC model, androgen responsive LNCaP PCa tumors are established in nude mice and treated with androgen ablation by castrating the tumor bearing mouse. This leads to the progression and adaptation of tumors to low androgen levels by synthesizing androgens *de novo*, and eventually to the castration-resistant stage (Locke et al., 2008).

Despite their utility, xenografts models with established cell lines also have significant and inherent limitations, e.g., by covering genetic changes after immortalization that are not representative of the disease. For example, PC3 and DU145 cells do not express PSA and little or no AR, which prevents studies of androgen signaling or castration sensitivity in these lines (Sobel & Sadar, 2005). The loss of AR and PSA is a common feature found in PCa cell lines produced from primary cultures of human PCa (Peehl, 2005), being an undesirable feature for modelling disease progression. It is noteworthy to state that a mouse prostate does not produce PSA, and serum PSA can, therefore, be used to measure tumor growth of PSA producing human PCa cells *in vivo* (Priolo et al., 2010).

2.6.1 Modelling castration-resistant prostate cancer

Androgen sensitive human PCa cell lines, such as LNCaP (Horoszewicz et al., 1980), 22Rv1 (Sramkoski et al., 1999), LAPC (Klein et al., 1997), DUCaP (Lee et al., 2001) and VCaP (Korenchuk et al., 2001), have been used both in cell cultures and as xenografts in castrated immunodeficient mice to elucidate CRPC mechanisms and in drug testing. These all have the characteristics typical of CRPC, but differ in e.g., tumorigenicity, growth rate, metastatic ability, and gene expression (Sampson et al., 2013).

Many PCa gene expression studies have been performed with the LNCaP cell line, that is derived from a lymph node metastasis (Horoszewicz et al., 1980). It has retained several key features of human PCa: the cells are of epithelial origin, and express both AR and AR-regulated genes, produces PSA protein, and are sensitive to androgen for growth and survival in culture and as xenografts (Dehm & Tindall, 2006). However, LNCaP cells have T878A (previously reported in the literature as T877A) mutation in AR that also binds steroid hormones such as P_4 and estrogens (Chen et al., 2015; Steketee et al., 2002), while this mutation has been identified in only a subset of patients. Various derivatives have been generated from this cell line to model PCa cell transformation to androgen-independency, including C4-2, which is able to form colonies on soft agar and grows in castrated mice (Wu et al., 1994).

The VCaP PCa cell line is derived from spinal bone metastasis in a CRPC patient, and the cells express wild-type AR as well as AR-regulated genes such as PSA and TMPRSS2-ERG fusion gene (Korenchuk et al., 2001; Loberg et al., 2006). In addition, in VCaP cells *in vitro* and in VCaP xenografts *in vivo*, AR mRNA levels decrease rapidly by androgen stimulation and increase in response to androgen depletion (Cai et al., 2009). According to our studies and experience, VCaP tumor xenografts have a good tumor take rate in intact immunodeficient mice, respond to castration with a reduced growth rate, and re-grow. The xenografts show after castration AR overexpression, regulation of AR splice variants and the activation of intratumoral androgen biosynthesis (Huhtaniemi et al., 2018; Knuuttila et al., 2014; Knuuttila et al., 2018), all also being classical features in clinical CRPC.

The main advantages of the cell line models are their relative simplicity, ease, and reproducibility, and allowing simultaneous testing of several compounds *in vitro* and *in vivo*. A good and successful example of the use of models in preclinical testing are clinically used enzalutamide and darolutamide, which were tested during the development on LNCaP and VCaP cell lines and xenograft models (Moilanen et al., 2015; Tran et al., 2009).

3 Aims

PCa is the second most common cancer and the fifth leading cause of death due to cancer in men worldwide (Bray et al., 2018). In the Nordic countries, one in five men will be diagnosed with PCa (Danckert et al., 2019) and in about 20% to 30% patients PCa metastasize (Han et al., 2003) and will progress to the lethal form, CRPC. Androgens and AR are essential regulators of normal male physiology and health, but they are also regulators of PCa cells even in CRPC.

The use of the VCaP xenograft model to study the progression and treatment modalities have provided valuable information about the CRPC growth regulation. The role of adrenal androgens as a driver for CRPC growth in a man is generally accepted, while the value of preclinical mouse models of CRPC has been questioned, due to the assumption that mouse adrenals do not produce steroids activating the AR.

Our aims have been to define the value of VCaP xenograft model to study CRPC growth and outline the antiandrogen resistance mechanisms. Our hypothesis has been that androgen-dependent growth plays an active role also in the late stages of PCa. We have done four large xenograft studies and collected large number of samples from which we have analyzed steroid concentrations and performed transcriptomics and histopathological analyses to study treatment responses.

The specific aims of this thesis were:

- To define the value of mice with VCaP xenografts as a preclinical model of CRPC by evaluating endocrine responsiveness of the model.
- To define anti-androgen resistance mechanisms in CRPC using the preclinical VCaP xenograft model *in vivo*.

4 Materials and Methods

Intact, orchiectomized (ORX), ORX and enzalutamide treated (ORX+Enza) and ORX and adrenalectomized (ORX+ADX) Athymic Nude male mice were used to define the role of adrenals to the castration- and antiandrogen resistant growth of VCaP PCa xenografts in mice.

4.1 Xenograft studies

The following Table 3 lists all the main information from the *in vivo* studies that have been carried out for this Thesis.

Table 3. Studies 1–3 numbered in chronological order. The table lists the number of inoculated cells and the inoculation sites, treatment groups, numbers of animals, administered substances or treatments, and license numbers of The National Animal Experiment Board of Finland. Abbreviations: subcutaneous (*s.c.);* orthotopic (*o.t.);* orchiectomy (ORX); adrenalectomy (ADX); enzalutamide (Enza).

STUDY	VCaP INOCULATIONS	TREATMENT GROUPS AND NUMBER OF ANIMALS USED (n)	LICENCE
1. Thesis I (Huhtaniemi et al., 2018)	Two million cells per mice to *s.c.*	1. Intact (n=15) 2. ORX (n=14) 3. ORX+ADX (n=14)	7472/04.10.03/ 2012
2. Thesis I (Huhtaniemi et al., 2018)	Two million cells per mice to *s.c.*	1. ORX (n=13) 2. ORX+ADX (n=13) 3. ORX+ADX+Corticosterone 3 mg/kg (n=13)	1993/04.10.03/ 2011
3. Thesis II (Huhtaniemi et al. 2022)	Two million cells per mice to *s.c.*	1. ORX I (n=17) 2. ORX II (n=17) 3. ORX+Enza I 20 mg/kg (n=14) 4. ORX+Enza II 20 mg/kg (n=14) 5. ORX+ADX I (n=15) 6. ORX+ADX II (n=13)	4199/04.10.07/ 2014

4.1.1 Cell culture

VCaP cells were obtained in 2006 from the American Type Cell Culture (ATCC, Manassas, VA, USA) and were tested and authenticated by short-tandem repeat analysis. The cells were cultured in RPMI-1640 medium supplemented with 4 mM Glutamax-I, 10% fetal bovine serum (FBS) and 1% penicillin (10,000 U/ml) and streptomycin (10 000 µg/ml). Cells were split 1:2–1:3 once a week by treating with 0.25% trypsin, and medium was changed at least twice a week. Four to 6 days before inoculation, the cells were divided to confirm optimal growth potential in mice. In studies 1 to 3 all cell culture reagents were purchased from Gibco™, Thermo Fisher Scientific, except HyClone™ fetal bovine serum was obtained from GE Healthcare, (Marlborough, MA, USA).

At the day of inoculation, the cells growing at 80% confluence were harvested using 0.05% Trypsin-EDTA. Harvested cells were centrifuged, counted and suspended in the culture medium described above. In studies 1 to 3, two million cells in 150 µl in combination with high protein concentration Matrigel™ [(1:1), BD Biosciences, Bedford, MA, USA], were inoculated *s.c.* into the right flank of each mouse using a 25 G needle at a density of 13.3 x10^6 cells/ml.

4.1.2 Animals

Athymic Nude male mice (Hsd:Athymic Nude-*Foxn1*^{nu}, Envigo, Gannat, France) at approximately 4–6 weeks of age, weighing between 20–30 g, were used in all studies. The mice were housed in individually ventilated cages (IVC, Techniplast, Buguggiate, Italy) under controlled conditions of light (12 hours light /12 hours dark), temperature (22±2 ºC) and humidity (55±15%) in specific pathogen-free conditions at the animal facilities of Orion Corporation (Orion Pharma, Turku, Finland) and at the Central Animal Laboratory, University of Turku, Finland. The mice were given irradiated soy-free natural-ingredient feed [RM3 (E), Special Diets Services, Essex, UK] and filtered UV treated or autoclaved tap water *ad libitum*. In order to maintain sodium balance, ORX+ADX mice had unlimited access to 0.9% sodium chloride (Baxter, Deerfield, IL, USA), instead of water.

The development and growth of the VCaP tumors were monitored by measuring the tumor volume twice a week, and by detecting the serum concentration of prostate specific antigen (PSA) every 7 or 10 days. The volume of the *s.c.* tumors were calculated according to following formula: W^2 x L / 2 (W = shorter diameter, L = longer diameter of the tumor). For PSA analysis, approximately 100 µl of blood was collected from the saphenous vein, and the PSA was measured from serum with an in-house time-resolved fluorometric assay (Lovgren et al., 1996). After blood collection, mice were injected *s.c.* with 200 µl of 0.9% NaCl (Baxter).

Animals were restrained during cell inoculations, blood sampling, weighing, and administering of the vehicle or test compounds. At the end of the studies, mice were

sacrificed, and blood, tumor, adrenal glands and other tissues were dissected out and collected for further use. Half of the tumor was fixed in 10% formalin for paraffin embedding and the rest was frozen at -80 ºC for further use.

4.1.3 Experimental design

In study 1 (I) intact, orchiectomized (ORX), orchiectomized and adrenalectomized (ORX+ADX) Athymic Nude male mice were used. Tumors were grown for 7 weeks, until the mean volume of the tumors reached approximately 300 mm^3 (range 51–691 mm^3), and the mean serum PSA value was approximately 20 µg/l (range 1–93 µg/l). The experimental groups of mice, intact (n=15), orchiectomized (ORX; n=14), and adrenalectomized and orchiectomized (ORX+ADX; n=14), were allocated to study groups using a matching model algorithm (Laajala et al., 2016) which matches baseline PSA concentration, tumor volume, PSA change from the previous week, and animal weight. This order represents the decreasing importance of the variable in the algorithm used. Following the ORX and ADX, the tumor volumes and the serum PSA were measured for eight subsequent weeks. At the end of the experiment the mice were sacrificed, and serum, adrenal glands and tumors were collected for further analyses. Samples for steroid measurements and for RNA isolation were stored at -80 ºC after initial freezing them in liquid nitrogen.

In study 2 (I) the VCaP tumors were initially established as described at study 1 in intact mice. When the tumors reached approximately 300 mm^3 (range 46–610 mm^3) the mice were assigned in tree groups: ORX (n=13), ORX+ADX (n=13) and ORX+ADX with corticosterone treatment (n=13). ORX and ADX were carried out as described above, and after operations, corticosterone (Sigma-Aldrich, St. Louis, US) was provided orally for four weeks (3 mg/kg/day) until the end of the study. The dose of corticosterone was selected based on literature and verified by the measuring of plasma ACTH levels at the time of necropsy, being 24 h after the last dose of corticosterone (Milliplex MAP Rat Pituitary Magnetic Bead Panel, Millipore Corporation, Billerica, MA, USA). At the end of the study, adrenal glands and tumors were dissected and snap-frozen, blood samples were collected by cardiac puncture, and serum was separated and stored in -80 ºC.

In study 3 (II), the castration-resistant *s.c.* VCaP xenografts were generated, as previously described in study 1. Tumors were grown for five weeks, until the mean volume of the tumors reached approximately 500 mm^3 (range 211–1554 mm^3), and the mean serum PSA value was 12 µg/l (range 1.4–53.8 µg/l). Mice were allocated to study groups: orchiectomized (ORX; n=34), orchiectomized and Enza treated (ORX+Enza; n=28), and orchiectomized and adrenalectomized (ORX+ADX; n=28) mice. Half of the mice were sacrificed during the treatment response and the rest when the treatment was no longer responding, and tumors became resistant.

4.1.4 Orchiectomy, adrenalectomy and antiandrogen treatments

Mice were castrated (orchiectomized, ORX) by removing testes through a scrotal incision. Adrenalectomy (ADX) was done through midline incision to the abdominal cavity and by entering through the abdominal wall, both sides of the dorsal incision. Orchiectomy and adrenalectomy were carried out under the isoflurane (2–3%, Baxter, Belgium) via induced anesthesia. For analgesia, mice were injected *s.c.* with buprenorphine (0.1 mg/kg, Temgesic® 0.3 mg/ml, Reckitt Benckiser Healthcare Ltd., Hull, UK) immediately, and two days after surgery with longer-acting carprofen 5 mg/kg (50 mg/ml Vet Rimadyl®, Pfizer SA, Louvain-La-Neuve, Belgium). Antiandrogen (20 mg/kg per day of enzalutamide) were administered orally via gavage once daily to mimic antiandrogen treatment in CRPC patients (Figure 6).

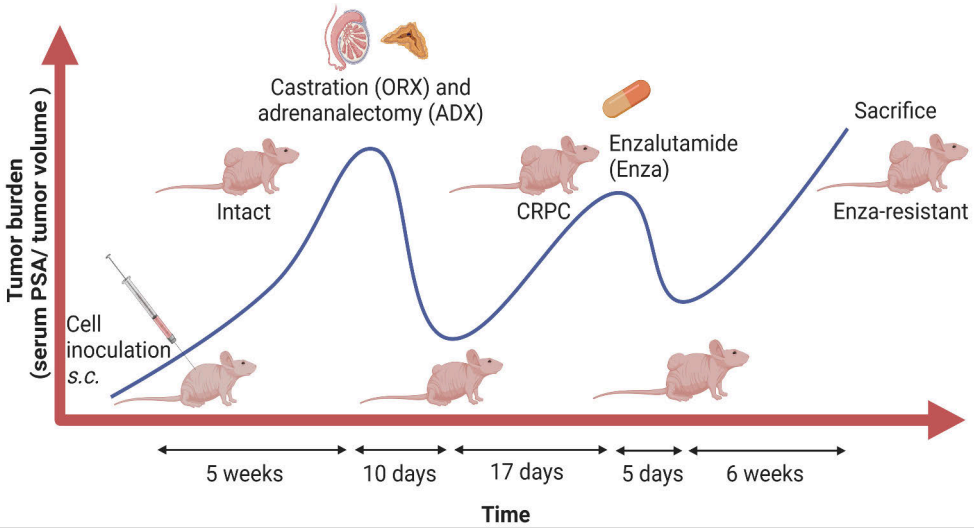

Figure 6. Schematic representation of the subcutaneous (*s.c.*) VCaP xenograft study course. Tumor burden was determined by measuring serum prostate-specific antigen (PSA) and tumor size. Orchiectomy (ORX), adrenalectomy (ADX) and enzalutamide (Enza) dosing were performed to model hormonal treatments of PCa. Created with BioRender.com

4.2 PSA measurements

Blood samples were collected from the medial vein of the hind leg (saphenous vein) into serum tubes (Microvette®, Sarstedt AG & Co., Germany). Serums were separated from blood after sampling and were kept at least 30 minutes at room temperature. Thereafter, serum was separated by centrifugation (1600 x G, 15 minutes, +4 °C) and frozen. Serum samples were stored at –80°C until analysis.

Analyses were carried out with an in-house method applying two antibodies in a direct sandwich technique and measured with time-resolved fluorometer (Viktor2 V 1420 Multilabel Counter, Wallac, PerkinElmer Analytical Life Sciences). Reagents for the PSA fluorometric assay were provided by Kim Petterson (University of Turku).

4.3 Steroid profiling

Tumors and adrenal glands were homogenized in sterile water using a TissueLyzer LT homogenizer (Qiagen, Venlo, The Netherlands), and intratumoral, intra-adrenal and serum concentrations of P_4, A-dione, T and DHT were measured using a previously described method applying GC-MS/MS (Nilsson et al., 2015). Using mouse serum, the lower limit of quantitation (LLOQ) for P_4, A-dione, T and DHT with the assay are 74 pg/ml, 12 pg/ml, 8 pg/ml, 2.5 pg/ml, respectively. Results under the LLOQ were calculated to be half of each LLOQ value to avoid overestimation of low values in the analysis. To make intra-adrenal and intratumoral steroid concentrations comparable to those in the serum, 1 g of adrenal and tumor was considered equivalent to 1 ml of serum.

4.4 Gene expression profiling

4.4.1 RNA and DNA extraction

Total RNA for RT-qPCR and RNAseq analyses were extracted from VCaP tumor and adrenal gland samples using TRIsure reagent (Invitrogen, Carlsbad, CA, USA), and purified using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Samples were homogenized using TissueLyzer LT and stainless-steel beads (Qiagen, Hilden, Germany) in RIPA-lysis buffer containing the following: 150 mmol/l Tris-HCl, 1% NP-40, 0,5% sodium deoxycholate, 1 mmol/l EDTA, 1 mmol/l SDS, 100 mmol/l sodium orthovanadate (Sigma-Aldrich, St. Louis, MO, USA), and cOmplete Mini protease inhibitor (Roche Diagnostics, Mannheim, Germany). RNA was DNase treated (Invitrogen) and quality determined using a fragment analyzer (Advanced Analytical Technologies, Ankeny, IA, USA).

4.4.2 RT-qPCR

The expression of the specific mRNAs of interest were analyzed through a reverse transcription quantitative polymerase chain reaction (RT-qPCR). The primers and conditions for RT-qPCR analysis for the expression of genes of interest are shown in the Table 4. Real-time monitoring of PCR amplification of cDNA was performed

using SYBR Green qPCR kit (Thermo Fisher Scientific, Waltham, MA, USA) and the target gene expression was normalized to the expression of ribosomal protein L19 (RPL19). The expression of mRNA was quantified using the Pfaffl method (Pfaffl, 2001).

Table 4. Real-time quantitative RT-PCR primer sequences.

HUMAN GENE	**FORWARD**	**REVERSE**
AKR1C2	5'-CCTAAAAGTAAAGCTCTAGAGGCCGT4 -3'	5'GAAAATGAATAAGATAGAGGTCAACATAG -3'
AKR1C3	5'- GCCAGGTGAGGAACTTTCAC -3'	5'- CAATTTACTCCGGTTGAAATACG -3'
AR-FL	5'- CTTACACGTGGACGACCAGA -3'	5'- GCTGTACATCCGGGACTTGT -3'
AR-V1	5'-CCATCTTGTCGTCTTCGGAAATGTTATGAAGC -3'	5'-CTGTTGTGGATGAGCAGCTGAGAGTCT -3'
AR-V7	5'-CCATCTTGTCGTCTTCGGAAATGTTATGAAGC -3'	5'- TTTGAATGAGGCAAGTCAGCCTTTCT -3'
FKBP5	5'- AAAAGGCCACCTAGCTTTTTGC -3'	5'- CCCCCTGGTGAACCATAATACA -3'
HSD17B3	5'- CTGAAGCTCAACACCAAGGTCA -3'	5'- CTGCTCCTCTGGTCCTCTTCAG -3'
KLK2	5'- CTGCCCATTGCCTAAAGAAGAA -3'	5'- GGCTTTGATGCTTCAGAAGGCT -3'
KLK3	5'- CCAAGTTCATGCTGTGTGCT -3'	5'- GGTGTCCTTGATCCACTTCC -3'
KLK4	5'- GGCACTGGTCATGGAAAACGA -3'	5'- TCAAGACTGTGCAGGCCCAGC -3'
NOV	5'- ACCGTCAATGTGAGATGCTG -3'	5'- TCTTGAACTGCAGGTGGATG -3'
PMEPA1	5'- TGCCGTTCCATCCTGGTT -3'	5'- AGACAGTGACAAGGCTAGAGAAAGC -3'
L19	5'- AGGCACATGGGCATAGGTAA -3'	5'- CCATGAGAATCCGCTTGTTT -3'
SRD5A1	5'- CCTGTTGAATGCTTCATGACTTG -3'	5'- TAAGGCAAAGCAATGCCAGATG -3'
SRD5A2	5'- CTCTCTAAGGAAGGGGCCGAAC -3'	5'- GACAATGCATTCCGCAAACATA -3'
ST6GalNAc1	5'- AGGCACAGACCCCAGGAAG -3'	5'- TGAAGCCATAAGCACTCACC -3'
SYTL2	5'- TCTGCCTTGAGAAAACAAACAGTT -3'	5'- GCCAGTGGGTGGCACTAAAA -3'
MOUSE GENE	**FORWARD**	**REVERSE**
Ar	5'- GTCTCCGGAAATGTTATGAA -3'	5'- AAGCTGCCTCTCTCCAAG -3'
Akr1c6	5'- CAGACAGTGCGTCTAAGTGATG -3'	5'- CGGATGGCTAGTCCTACTTCCT-3'
Akr1c18	5'- TGGCACTGTGAAAAGGGAAGAT -3'	5'- TTAGGCAAAAGCTCATTCCCTGG -3'
Akr1d1	5'- TTGCGTTTCAACATCCAGCG -3'	5'- AGCAACTCCACATAGCGGAC -3'
Cyp11a1	5'- AGATCCCTTCCCCTGGCGACAATG -3'	5'- CGCATGAGAAGAGTATCGACGCATC -3'
Cyp17a1	5'- CAAGCCAAGATGAATGCAGA -3'	5'- AGGATTGTGCACCAGGAAAG -3'
Hsd3b1	5'- CAGGAGCAGGAGGGTTTGTG -3'	5'- GTGGCCATTCAGGACGAT -3'
Hsd3b2	5'- CAGTTGTTGGTGCAAGAGGA -3'	5'- CCTGGGAATGACACCTGTGA -3'
Hsd17b3	5'- CACGGGGATAAAGACCAGGT -3'	5'- GATCGCAGGAAAGAGCTTGG -3'
Hsd17b6	5'- TTTGGAGGATTCTACAGTTGCTC -3'	5'- TCACCCCGAAATCTTGAACCT -3'
L19	5'- GGACAGAGTCTTGATGATCTC -3'	5'- CTGAAGGTCAAAGGGAATGTG -3'
Lhcgr	5'- GCCCTGAGCCCTGCGACTGC -3'	5'- AAAGCGTTCCCTGGTATGGTGGTT -3'
Mc2r	5'- TCTGACATCATGTTGGGCAGTCT -3'	5'- TGGTGATGTAACGGTCAGCT -3'
Srd5a1	5'- TGAGCCAGTTTGCGGTGTAT -3'	5'- CTCCACGAGCTCCCCAAAAT -3'
Srd5a2	5'- CACAGACATGCGGTTTAGCG -3'	5'- AACAAGCCACCTTGTGGGAT -3'
Srd5a3	5'- CTGGCTTAGTGCTCTGCTCA -3'	5'- CACAACGTGAATGGCTGCAT -3'
Ugt1a1	5'- GCAGAGTGGTTTATTCCCCCT -3'	5'- AGGCGTTGACATAGGCTTCAA -3'

4.4.3 RNA sequencing

The RNA-seq analyses of intact (n=5) and ORX (n=6) adrenal glands from study 1 and tumors of ORX mice treated for 5 days (ORX I, n=10) or 46 days (ORX II, n=10) with Enza from study 3, was carried out at the Finnish Functional Genomics Centre (University of Turku, Åbo Akademi University and Biocenter Finland). The samples were treated to according to Illumina TruSeq® Stranded mRNA Sample Preparation Guide and sequenced with Illumina HiSeq 2500 instrument (Illumina, San Diego, CA, USA) using Hiseq v2 Rapid sequencing chemistry and 50 bp single end read length.

The quality of the sequenced readings was checked using FastQC tool (Andrews, 2010). STAR v2.5.0c (Dobin et al., 2013) was used to align the readings to the mouse reference genome mm10, available at University of California, Santa Cruz Genome Bioinformatics Group (downloaded from Illumina iGenomes web site, San Diego, CA). The number of uniquely mapped reads associated with each gene according to RefSeq gene annotation was counted using the Subreads package (Liao et al., 2014). The downstream analysis of the data was performed using R version 3.2.2 (R Developement Core Team, 2015) and its corresponding Bioconductor module 3.2 (Gentleman et al., 2004). The count data was normalized for a library size using the Trimmed Mean of M-values (TMM) method implemented in edgeR package (Robinson et al., 2010). The normalized data was further transformed using the voom approach in the limma package (Ritchie et al., 2015). R package ROTS (Suomi et al., 2017) was used for performing the statistical testing of differentially expressed genes between the study groups. False discovery rate (FDR) < 0.05 and absolute fold change (FC) > 1.5 was required to consider the gene to be differentially expressed between the groups analyzed. The hierarchical clustering of the normalized expression values of differentially expressed AR-regulated genes was performed using Euclidean distance and Ward's method, implemented in the R package pheatmap (Kolde, 2015).

To analyze the role of AR signaling in our model, we collected a list of AR-related genes that contain known androgen-regulated genes as well as AR-interacting proteins using common up-regulated genes in VCaP and LNCaP after DHT treatment (Asangani et al., 2014), Database Resources of the National Center for Biotechnology (Coordinators, 2016), androgen pathway product analysis list provided by SwitchGear Genomics (Menlo Park, CA, USA) and Regulators of Androgen Action Resource (RAAR) database (Depriest et al., 2016).

4.4.4 DNA sequencing

To determine whether the AR mutations T878A, F877L and L702H promoted Enza resistance, we amplified AR exon 4 and beginning of exon 8 from cDNA samples

by using PCR (primers: AR exon 4 Se: ACAGGAGGAAGGAGAGGCTT; AR exon 4 As: CCCACTTGACCACGTGTACA; AR exon 7 Se: ACATCCTGCTCAAGACGCTT; AR exon 8 As: TGGGTGTGGAAATAGATGGGC). The amplified products were run on a 2% agarose gel. DNA containing gel pieces were extracted and purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. After that the DNA samples were prepared for sequencing with the Mix2Seq kit (Eurofins Genomics, Konstanz, Germany) and sequenced at Eurofins Genomics Sequencing Europe using cycle sequencing on ABI 3730XL machines (Applied Biosystems, Foster City, CA, USA).

4.5 Protein analyses

4.5.1 Western blot analysis

Tumor samples were homogenized using a TissueLyzer LT and stainless steel beads (Qiagen, Hilden, Germany) in radioimmunoprecipitation assay (RIPA)-lysis buffer [150 mmol/l Tris-HCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mmol/l EDTA, 1 mmol/l SDS, 100 mmol/l sodium orthovanadate (Sigma-Aldrich, St.Louis, MO, USA), and cOmplete Mini protease inhibitor (Roche Diagnostics, Mannheim, Germany). Samples (30 µg) were centrifuged at 8000 g for 10 minutes at 4 °C, and total protein concentrations in the supernatant were measured with a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

The protein samples were loaded onto a 10% Mini-PROTEAN® TGX™ Precast Protein SDS-PAGE Gel (Bio-Rad Laboratories, Hercules, CA, USA) and transferred onto an Amersham Hybond P 0.45 PVDF blotting membrane (GE Healthcare Life Sciences, Chicago, IL, USA) using a Trans-Blot Turbo transfer system (Bio-Rad Laboratories). The membranes containing tumor samples, were probed with rabbit monoclonal anti-AR antibody (dilution 1:2000, Abcam, Cambridge, UK) followed by HRP-linked anti-rabbit IgG secondary antibody (dilution 1:5000, Cell Signaling Technology, Denver, USA). Detection of specific bands was done using Cy5 and Cy3 detection and a Typhoon laser scanner (GE Healthcare) and imaged with an ImageQuant LAS 4000 camera system (GE Healthcare). ImageJ software version 1.51K (NIH, Bethesda, MD) was used to compare and quantify the intensity of bands on scanned images of membranes.

4.5.2 Immunohistochemistry

VCaP tumors and adrenal glands from xenografts were used for immunohistochemical analysis. Formalin-fixed, paraffin-embedded samples were

cut to sections prior to deparaffinization and rehydration. The sections were exposed to the antigen retrieval in a steamer in 10 mM sodium citrate buffer (Citric acid monohydrate, Sigma-Aldrich, St Louis, USA and Tri-sodium citrate dehydrate (Merck, Darmstadt, Germany) for 30 minutes.

Tumor slides were incubated in a humidified chamber overnight with the primary antibody against the N-terminus of the AR (N-20 sc-816, dilution 1:250, Santa Cruz Biotechnology, Dallas, Texas, USA) at +4 °C. Slides of adrenal glands were incubated with antibodies against the CYP11A1 (C-16:sc-18043, dilution 1:100, Santa Cruz Biotechnology), CYP17A1 (N-18:sc-46085, dilution 1:50, Santa Cruz Biotechnology) and 17β-HSD3 (C-14: sc-66415, dilution 1:100, Santa Cruz Biotechnology). Endogenous peroxidase activity was blocked by applying 1% H_2O_2 for 20 minutes at room temperature and the section was then incubated for 30 minutes with anti-rabbit antibody conjugated with polymer-HRP (Dako, Glostrup, Denmark), washed and visualized with Envision™+ System-HRP DAB staining (Dako).

Sections were counterstained with Mayer's hematoxylin (J.T Baker, Deventer, Netherlands), dehydrated with graded EtOH, rinsed with xylene and mounted with DPX (Merck). Slides were digitalized with a Pannoramic 250 slide scanner (3DHISTECH, Budapest, Hungary).

4.6 Statistical analyses

The statistical tests were chosen depending on the results of the preliminary Shapiro-Wilk tests for normality. Non-parametric Mann-Whitney, Kruskal-Wallis and Dunn's multiple comparison tests were applied for RT-qPCR comparison on single gene level, and to study the differences in the steroid concentrations of different treatment groups. These univariate statistical analyses were performed using GraphPad Prism 7 and 8 software (GraphPad Software, San Diego, CA, USA).

The longitudinal analysis of PSA and tumor size profiles between interventions was performed using mixed-effects models that infer differences in population growth slope co-efficient over the whole time of the intervention (Laajala et al., 2012), where the individual prognostic baseline variables are accounted for by incorporating them in the baseline matching (Laajala et al., 2016). The R statistical software (version 3.2.1) (R Development Core Team, 2016) together with the preclinical analysis R-package hamlet (version 0.9.5) (Laajala et al., 2016) were utilized in the longitudinal analyses in study 1 and study 2. In study 3 and 4 the Two-way ANOVA with Tukey's multiple comparisons test (GraphPad) were used to test differences in tumors grown. In all the studies $P < 0.05$ was considered statistically significant.

5 Results

5.1 Antiandrogen treated castration-resistant VCaP xenograft model exhibits features of clinical castration-resistant prostate cancer

Androgen-targeted therapy in men with PCa focuses on three different approaches to inhibit androgen signaling and cancer cell proliferation: 1) Chemical or surgical castration which suppresses testicular steroid synthesis, 2) inhibition of steroid synthesis and 3) blocking the AR with antiandrogens. The features of the animal model need to be comparable to clinical tumors and be clinically relevant. Therefore, we validated the model by analyzing hormonal responses typical to clinical tumors.

5.1.1 Castration and antiandrogens have a transient treatment effect on tumor growth

In all our studies, serum PSA and *s.c.* tumor size was used as markers of tumor growth as mouse prostate does not produce PSA but VCaP cells do.

In study 1 (I) of the present thesis, the development and growth of the *s.c.* VCaP tumors was monitored by measuring the tumor volume (I; Figure 1A) by calibers twice a week and by detecting the serum PSA concentrations every 10 days (I; Figure 1B). Intact tumors grew constantly and after ORX, the serum PSA dropped within 2 weeks, and thereafter increased again, and reached the pre-castration levels about 7 weeks after ORX. ORX also induced regression of the tumor growth and the tumor volume stayed stable almost for 3 weeks and thereafter, relapsed to regrowth.

In the study 3 (II) Enza response on VCaP tumors was transient, being effective for about 3 weeks, as measured by the tumor size (II; Figure 1B). Serum PSA measurements showed a similar pattern, with a more marked, but shorter treatment response time of only 5-10 days. After 14 days of Enza treatment the PSA level was higher than that observed before the treatment (II; Figure 1C).

In the study by Knuuttila et al. (2018), the effects of castration and antiandrogen treatment on the *o.t*. VCaP tumors growth were similar. PSA increased continuously in the intact mice during the 14-week follow-up period, and ORX (2 to 5 weeks after inoculation) caused a temporary one week decrease in PSA levels that started to

increase again after two weeks of ORX, indicating castration-resistant growth of the VCaP tumors (Knuuttila et al., 2018). In this study, mice bearing CRPC tumors were treated with antiandrogens (enzalutamide or apalutamide, 20 mg/kg) for 4 weeks that significantly reduced serum PSA with both antiandrogens, but no significant difference in *o.t.* tumor size observed at the end of the experiment (Knuuttila et al., 2018). As we expected, the identical growth pattern was observed in *s.c.* and *o.t.* VCaP tumors. Also, the growth response of ORX VCaP xenografts to enzalutamide (Enza) was similar than observed in study with *o.t.* model.

5.1.2 Decreased intratumoral androgens in response to castration and antiandrogen treatment

Since the castration and antiandrogens had treatment effects on tumor growth, we also studied levels of the AR ligands. Concentrations of P_4, A-dione, T and DHT in tumor, serum and adrenal gland samples were analyzed by gas chromatography-tandem mass spectrometry (GC-MS/MS) (Table 5). Detectable levels of all these steroids were measurable in the tumors of intact, ORX (CRPC) and ORX+Enza treated mice in studies 1 (I) and 3 (II).

In the study 1 (I), ORX decreased intratumoral T and A-dione concentrations, but did not affect the concentrations of P_4 and DHT (I; Figure 4B). In particular, ORX appears to have only a minor and transient effect on intratumoral DHT concentrations when comparing intact tumor concentrations to short-term (ORX I) and castration-resistant (ORX II) concentrations (Table 5). Noteworthy, intratumoral P_4 and androgen concentrations were high in castration-resistant VCaP tumors, despite low serum steroid concentrations in ORX mice in all studies (Table 5).

Short term Enza treatment (Enza I, five-day dosing period) in study 3 (II), decreased intratumoral T and DHT in the CRPC tumors, but during the long-term Enza treatment (Enza II, 47 days post dosing) concentrations increased back to the CRPC level (II; Figure 3C) (Note that the figure panels are wrongly referred to in the text of the publication II, but correctly referred to here). Enza treatment also had no effect on intratumoral or serum P_4 or A-dione concentrations, except that at the end of the experiment, serum A-dione level was elevated after long term Enza treatment compared to ORX alone effect.

Table 5. Concentrations of progesterone (P_4), androtenedione (A-dione), testosterone (T) and dihydrotestosterone (DHT) in tumor, serum and adrenal gland samples analyzed by gas chromatography-tandem mass spectrometry (GC-MS/MS) from studies 1 (I) and 3 (II). 1) Intact (n=10): 60-84 days post inoculations, 2) ORX I (n=17): 50 days post inoculations and 11 day post ORX, 3) ORX (n=14): 109 days post inoculations and 62 days post ORX, 4) ORX II (n=17): 117 days post inoculations and 78 days post ORX, 5) ORX+Enza I (n=14): 76 days post inoculations, 36 days post ORX and 5 days post enzalutamide (Enza), 6) ORX+Enza II (n=14): 116 days post inoculations, 36 day post ORX and 45 post Enza, 7) ORX+ADX I (n=15): 50 days post inoculations and 61 days post ORX+ADX operations, 8) ORX+ADX (n=13): 109 days post inoculations and 61 post operations, 9) ORX+ADX II (n=11): 117 days post inoculations and 76 day post operations, 10) ORX+ADX I no tumor (n=17): 9 day after operations, 11) ORX+ADX II no tumor (n=15): 76 day after operations.

Study groups	TUMOR mean (±SEM) (pg/g)				SERUM mean (±SEM) (pg/ml)				ADRENAL GLAND mean (±SEM) (pg/g)			
	P_4	A-dione	T	DHT	P_4	A-dione	T	DHT	P_4	A-dione	T	DHT
1 Intact (Study 1)	3513 ±1606	649 ±198	9000 ±1406	3902 ±453	754 ±409	91 ±36	318 ±1341	76 ±36	4 x10^6 ±1 x10^6	1711 ±330	2957 ±1340	1488 ±229
2 ORX I (Study 3)	434 ±161	37 ±5	552 ±193	2636 ±439	263 ±80	30 ±2	3 ±1	3 ±1	3 x10^6 ±3 x10^5	3505 ±1272	2172 ±1316	1015 ±114
3 ORX (Study 1)	1090 ±441	97 ±22	450 ±76	4633 ±823	305 ±81	9 ±1	11 ±3	8 ±2	5 x10^6 ±1 x10^6	2845 ±510	624 ±99	1034 ±201
4 ORX II (Study 3)	424 ±121	33 ±4	937 ±185	4363 ±713	370 ±115	13 ±2	18 ±7	7 ±3	2 x10^6 ±4 x10^5	4205 ±875	3417 ±1277	2371 ±427
5 ORX+Enza I (Study 3)	77 ±36	52 ±6	12 ±5	122 ±28	415 ±138	25 ±3	5 3	6 ±4	2 x10^6 ±2 x10^5	3135 ±879	1116 ±348	1539 ±368
6 ORX+Enza II (Study 3)	139 ±74	55 ±7	446 ±79	2045 ±378	287 ±91	31 ±3	26 ±6	20 ±5	1 x10^6 ±2 x10^5	12154 ±3723	9020 ±2865	4923 ±782
7 ORX+ADX I (Study 3)	20 ±7	9 ±1	521 ±170	1153 ±166	26 ±8	15 ±3	4 ±2	1 ±0	NA	NA	NA	NA
8 ORX+ADX (Study 1)	52 ±22	63 ±48	80 ±15	532 ±104	23 ±8	4 ±1	0 ±0	1 ±0	NA	NA	NA	NA
9 ORX+ADX II (Study 3)	17 ±3	19 ±6	695 ±382	741 ±248	19 7	17 ±2	6 ±4	0 ±0	NA	NA	NA	NA
10 ORX+ADX I no tumor (Study 3)	NA	NA	NA	NA	45 ±17	15 ±2	2 ±1	0 ±0	NA	NA	NA	NA
11 ORX+ADX II no tumor (Study 3)	NA	NA	NA	NA	56 ±19	38 ±5	1 ±0	0 ±0	NA	NA	NA	NA

The intratumoral concentrations for all the steroids measured in studies 1 (I) and 3 (II) during ORX and Enza treatment were higher than in serum, indicating local biosynthesis, furthermore serum androgen concentrations did not follow the concentrations measured in the tumors. The potential contribution of mouse stromal cells into intratumoral androgen synthesis was also studied in study 3 (II) by analyzing the expression of steroidogenic enzymes in tumor samples by RT–qPCR

using primers specific for mouse transcripts. However, the data revealed that classical steroidogenic enzymes such as CYP11A1, CYP17A1, HSD3B1 and -2, HSD17B3 and SRD5A1 and -2 were not considerably expressed in the mouse stromal compartment. Thus, high androgen levels in Enza resistant tumors suggest a role for AR-mediated treatment resistance.

5.1.3 Castration and antiandrogen affect AR and AR related gene expression

To understand the potential AR action in VCaP tumors, we have analyzed AR expression of different studies and treatment groups by using RT-qPCR, RNA-sequencing, immunoblotting, and immunohistochemistry.

Expression of full-length androgen receptor (AR-FL) and main splice variants AR-V1 and AR-V7 mRNA and AR protein levels were increased in *s.c.* VCaP tumors by ORX in study 1 (I; Figure 5A). In our previous study with *o.t.* tumors, four weeks antiandrogen treatment increased the mRNA expression of AR-FL, AR-V1, AR-V7 and AR protein expression (Knuuttila et al., 2018), but the same effect was not seen with 47 days Enza treatment in study 3 (II) were these were expressed very similarly in the tumors of the different treatment groups (II; Figure 2A–D). However, immunohistochemical studies revealed that after ORX, AR was localized to the nucleus in the presence of the antiandrogens in study 3 (II), as in study by Knuuttila et al. (2018) with o.t. VCaP tumors (Figure 7).

In our previous study (Knuuttila et al., 2018) we found interestingly two genes, up-regulated gene nephroblastoma overexpressed (NOV) and most down-regulated gene ST6 N-Acetylgalactosaminide Alpha-2,6-Sialyltransferase 1 (ST6GalNAc1), that could be considered as a novel sensitive marker for antiandrogen action. In study 1 (I) the relative mRNA expression of these genes was similar (I; Figure 5D) but with the longer exposure of Enza, in experiment 3 (II), the NOV gene was most down-regulated (II; Table 1) and the ST6GalNAc1 was not among the significantly changed androgen receptor regulated genes.

To understand the mechanism of the increased intratumoral steroid levels after castration and antiandrogen (ORX+Enza I vs. ORX+Enza II) treatment, we studied expression of steroidogenic enzymes in the tumors. Expression levels of traditional steroidogenic enzymes were not significantly elevated in Enza-resistant tumors in study 3 (II), when comparing Enza-responsive (ORX+Enza I) and Enza-resistant (ORX+Enza II) tumors. Of the 124 genes, potentially related to steroid synthesis, (SDR, AKR, and CYP enzymes) analyzed, only 8 genes (CYP1B1, SLCO2A1, CYP4F62P, SULT1C4, CYP4F8, CYP4F30P, FASN and RDH11) had significantly altered expression level. This result did not explain to us a possible steroid synthesis pathway in Enza-resistant tumors.

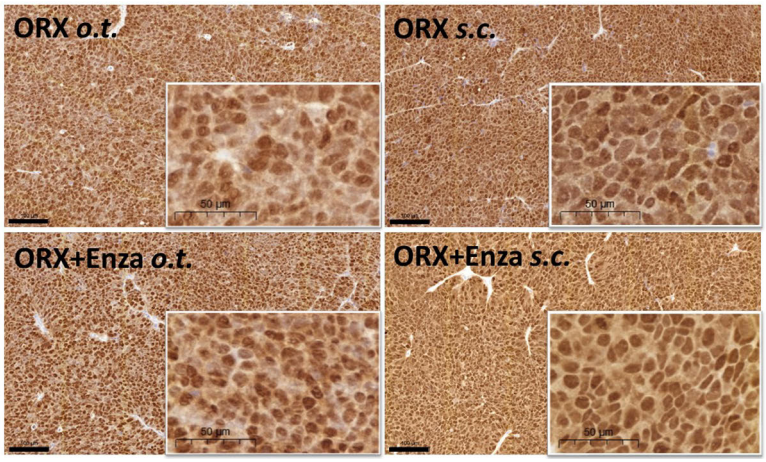

Figure 7. Representative figures of castrated (ORX) orthotopic (*o.t.*) and subcutaneous (*s.c.*) VCaP tumors with and without enzalutamide (Enza) treatments. Immunohistochemical staining revealed that after ORX, in castration resistance, AR was localized to the nucleus even in the presence of the antiandrogen Enza in *o.t.* and *s.c.* studies.

5.2 Growth of castration-resistant VCaP tumors is partially adrenal driven

Already in study by Knuuttila et al. (2018) with VCaP *o.t.* tumors, we found that VCaP tumors were not the major source of circulating androgens because serum androgen levels did not follow the concentrations measured in tumors. Since part of an androgen in humans is derived from the adrenal glands, we wanted to determine if it is also possible in our xenograft model, contrary to the current understanding of rodent adrenal gland and androgen synthesis.

5.2.1 Adrenalectomy combined with castration is more efficient than castration alone

In study 1 (I) combined ORX and ADX treatment provoked the serum PSA to drop from the pre-castration level of 20 µg/l to close to the limit of detection within 2 weeks (I; Figure 1B). Thereafter PSA levels, increased again but did not reach the pre-castration levels within the 9 weeks study period after operations. In addition, tumor volume of ORX+ADX treated mice stayed stable longer than in mice in ORX alone (I; Figure 1A). However, after 5 weeks ORX+ADX tumors relapsed to a growth phase. Thus, this showed that ADX significantly reduces the growth of the *s.c.* CRPC VCaP tumors, measured both by tumor volume and by serum PSA, indicating a significant contribution of the adrenal gland to the CRPC growth in mice. Interestingly, castration significantly increased adrenal weight compared to intact adrenals. RNA-seq data of adrenal glands from intact and ORX-treated mice

were also well divided into two distinct clusters with 135 genes up-regulated and 61 down-regulated (Figure 8).

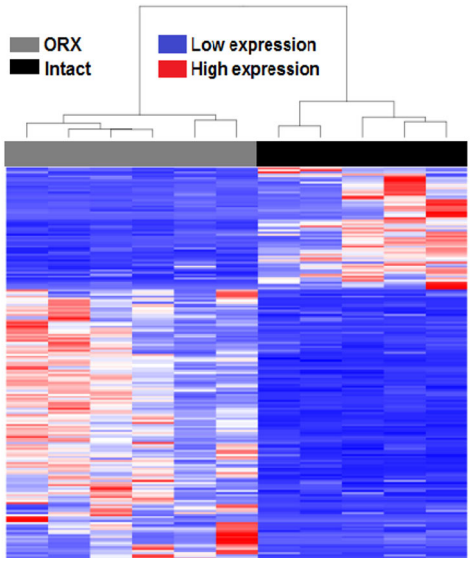

Figure 8. The heat map clustering of the differentially expressed (DE) genes in mouse adrenal gland for the comparison. The clustering is based on the general expression measurement similarity. In the plot red color means high expression and blue low expression. Each row represents one DE genes and each column represents one sample (ORX=Grey, Intact=Black) Previously unpublished data.

In order to further test the contributing adrenal factors affecting the CRPC growth we provided the ORX+ADX mice carrying CRPC tumors with a corticosterone treatment in study 2 (I). However, our results showed that the glucocorticoid replacement (corticosterone) did not affect the tumor growth. Serum levels of ACTH were elevated in ORX+ADX mice and corticosterone treatment appeared to restore it to almost the same level as in ORX mice through its negative feedback effect. This demonstrated that the drop in the adrenal driven corticosteroid production does not contribute to the reduced growth after ADX.

5.2.2 Mouse adrenals express enzymes involved in androgen production

Because glucocorticoid deficiency did not explain the inhibition of VCaP xenograft growth in the ORX+ADX group in study 2 (I), we analyzed the expression of adrenal enzymes involved in sex steroid biosynthesis by RNAseq, RT-qPCR analyzes and immunohistochemistry in study 1 (I).

Several enzymes and proteins essential for *de novo* synthesis of androgens were present in mouse adrenals. RNAseq unexpectedly suggested several potential pathways for androgen production in adrenal glands due to the significantly increased mRNA expression of several CYP, AKR, and SDR family enzymes mRNA expression, including both classical steroid biosynthesis enzymes and several other enzymes not yet known to be involved in steroid synthesis (I; Figure 2D). Some of these mRNA levels of enzymes were confirmed using qRT-PCR analysis (I; Figure 2A-B). Of those, the expression of classical enzymes *Cyp11a1*, *Cyp17a1*, *Hsd3b1*, *Hsd3b2*, *Srd5a1,* and *Srd5a3* were significantly induced by ORX as compared with the adrenals of the intact mice. Interestingly e.g., *Akr1c18* were shown to be induced even over 3700-fold by ORX. Orchiectomy (ORX) also caused adrenal weight gain and hypertrophy in mouse adrenal HE-stained cells and induced the expression of CYP11A1 and CYP17A1 enzymes also in immunohistochemical stainings (Figure 9).

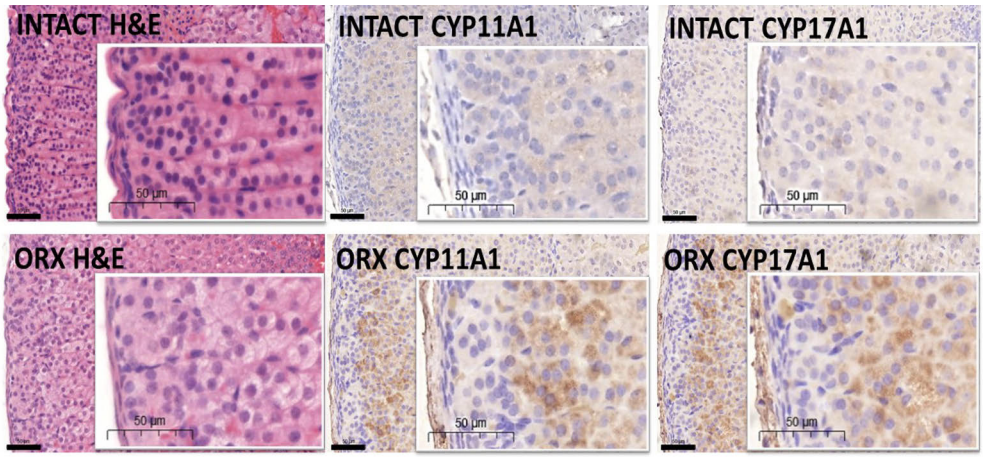

Figure 9. Orchiectomy (ORX) causes hypertrophy in hematoxylin and eosin (HE) stained cells of mouse adrenal gland and induces the expression of CYP11A1 and CYP17A1 enzymes, which was detected among other analysis by immunohistochemistry. Previously unpublished data.

In addition, ORX resulted in significant up-regulation of the luteinizing hormone/ choriogonadotropin receptor (*Lhcgr*), melanocortin 2 receptor (*Mc2r*), and *Ar* mRNA expression in mouse adrenal glands (I; Figure 2C). Furthermore, a significant correlation between *Cyp17a1* and *Lhcgr* expression was also observed, suggesting that circulating LH induced by ORX can effectively prompt the early stages of adrenal steroidogenesis (Figure I; 2E).

5.2.3 Mouse adrenals produce androgens and their precursors

Since the growth of the *s.c.* castration-resistant VCaP tumors is partially adrenal driven and there is an enzymatic machinery for steroid synthesis in the mouse adrenal gland, we were interested in the intra-adrenal steroid concentrations in intact and ORX male mice.

Interestingly, in study 1 (I) high concentrations of all main classic androgens (A-dione, T and DHT) and P_4 were present in the adrenals of intact and ORX mice, of which P_4 concentrations were over 1000-fold higher than other steroids. In addition, high tissue / serum ratios of A-dione, DHT and P_4 also support local adrenal steroid synthesis. Removal of adrenals reduced intratumoral and serum P_4 and DHT concentrations, as well as serum T levels, indicating circulatory steroids originating from the adrenal glands. An important finding was also that ORX alone had no effect on serum or intratumoral P_4 concentrations, proving that P_4 mainly originates from the murine adrenals.

At the same study 1 (I), adrenal steroids were also found to influence intratumoral A-dione, T, DHT and P_4 steroid concentrations being decreased after ADX in ORX mice. However, the tumor / serum ratio of DHT remains high after ADX, also suggesting tumor local steroid synthesis. In conclusion, our data indicate that in contrast to the current view, and similar to human, mouse adrenals synthesize significant amounts of steroids that contribute to the AR dependent growth of CRPC demonstrated by VCaP xenograft.

5.2.4 Adrenalectomy affects AR and androgen-dependent gene expression in the CRPC VCaP tumors

In study 1 (I), the intratumoral androgen production and expression of full-length AR and AR splice variants AR-V7 and AR-V1 was induced in the CRPC tumors (I; Figure 5A). After the removal of circulating androgens by ORX and ADX, the VCaP tumors reappear and present with high expression of full-length AR and express AR splice variants. In studies 1 (I) and 3 (II), ADX significantly further induced the expression of full-length AR and the AR-V7 and AR-V1 variants in the VCaP tumors.

The expression of a set of genes previously identified to be regulated by antiandrogens (Knuuttila et al., 2018), were analyzed part of them with RT-qPCR also in study 1 (I). Of these genes for example NOV were up-regulated and ST6GAlNAc1 were down-regulated after ORX+ADX, giving the idea that the ADX effect was similar to that shown for antiandrogens in our earlier study (Knuuttila et al., 2018). The classical marker genes of androgen action such as the various kallikreins were not significantly changed between the tumors grown in the ORX

and ORX+ADX mice in study 1 (I; Figure 5D). In contrast, the enzymes AKR1C3 and SDR5A1 involved in DHT synthesis were induced after ORX+ADX (I; Figure 5C).

5.3 Long exposure to antiandrogen leads to therapy resistance

To find out how antiandrogen resistance develops during the Enza treatment, we conducted the study 3 and sacrificed half of the mice during the Enza treatment response and the rest when the treatment was no longer responding and became resistant. In line with our previous study (Knuuttila et al., 2018), Enza had an effect only two-three weeks and then tumors started to became treatment resistant. It is also noteworthy that we previously thought that antiandrogen treatment is similar than ORX+ADX effect, but study 3 data showed the effect of ORX+ADX on tumor growth was more stable compared ORX+Enza treatment.

5.3.1 AR action is regulated by AR and androgens

Our earlier study indicated a further increase in AR-FL, AR-V1, and AR-V7 mRNA and protein expression after ORX and antiandrogen treatment compared with the ORX and vehicle-treated mice (Knuuttila et al., 2018). In longer treatment study 3 (II), mRNA and protein levels of full-length AR and splice variants were expressed very similarly in different treatment groups, except there was significant up-regulation of all *AR* mRNA forms after long exposure ORX+ADX tumors (II; Figure 2A, C–D). Immunohistochemical staining revealed cytoplasmic localization of AR during the treatment responses and more nuclear staining after long exposure of ORX and ORX+Enza (II; Figure 2E). Interestingly in long exposure of ORX+ADX there was also AR negative cells, and not so clear nuclear localization of AR (II; Figure 2E).

In RNA-seq data, full-length AR was strongly expressed in both Enza-responsive (ORX+Enza I) and Enza-resistant (ORX+Enza II) tumors, suggesting a central role for AR during CRPC and antiandrogen therapy. The role of AR as a promoter of Enza resistance is further reinforced by the fact that in Enza-resistant tumors, AR had the fourth highest expression level of the entire transcriptomic (CPM, counts-per-million). In addition, of the AR regulated genes 30 genes were significantly up-regulated and 2 genes were down-regulated between Enza I and Enza II groups. These results confirmed us that AR signaling is still active during the Enza resistance.

We also investigated whether common AR mutations would explain the maintenance of AR signaling in Enza-treated tumors (Enza I and Enza II) by

analyzing tumor samples for the common mutations T878A, F877L, and L702H found in CRPC patients. However, none of these samples contained these AR mutations, suggesting that the function of AR in these VCaP tumors is normal and driven by androgens.

5.3.2 Reduced amount of intratumoral androgens is a transient response to antiandrogen treatment

Our previous study with *o.t.* tumors demonstrated that a 4-week long treatment with antiandrogens significantly reduced intratumoral T and DHT levels in the castration-resistant VCaP xenografts compared with ORX+vehicle treated mice (Knuuttila et al., 2018). Similarly, in study 3 (II), low levels of T and DHT was observed in the tumors during the short Enza (ORX+Enza I) treatment response (II; Figure 3C). However, in those tumors that had escaped the treatment response (ORX+Enza II) the T and DHT concentration restored to the level observed in the vehicle treated ORX tumors (II; Figure 3C). Notably, T and DHT levels increased up to 38-fold and 17-fold, respectively, likely inducing AR-dependent growth even in the presence of Enza.

5.3.3 Antiandrogen resistance alters the tumor transcriptome expression

According to the RNA-seq results from study 3 (II) there was a clear bias toward induced gene expression in the Enza-resistant tumors. Enzalutamide resistance altered the tumor gene expression by affecting 292 genes significantly. From those, 230 genes were up-regulated, and 62 genes were down-regulated between Enza I and Enza II tumors (II; Figure 5A). Pathway analysis of differentially expressed genes between these tumors revealed terms such as inflammatory response, angiogenesis and vascular development and morphogenesis, and cell adhesion and mobility. Similar conclusions were found by PubMed search of the TOP 10 genes with the highest up-regulation and down-regulation in Enza II tumors compared to Enza I (II; Table S3). Most of these genes were associated with androgen action, while they were also strongly associated with proliferation, apoptosis, invasiveness, cell migration, EMT, and neuroendocrine development. Thus, the resistance mechanism is likely to involve several pathways that promote tumor progression.

We also analyzed the expression of AR-related genes in samples from the ORX+Enza I and ORX+Enza II groups and found that 253 of the 536 genes tested, were expressed in both groups in the study 3 (II). The difference between these groups was analyzed by hierarchical clustering using AR-related genes (The selection of AR-related genesis is described in section 4.4.3) of which 30 genes were

up-regulated and 2 genes were down-regulated in Enza-resistant tumors (II; Table 1). These changed genes included several well-characterized androgen-regulated genes such as KLK3, LOX, ELL2, FKBP5, TMPRSS2, and PMEPA (II; Figure 5E). Surprisingly, regulation of NOV expression was also found to be down-regulated. This result was not in line with our expectations, as in our previous studies the NOV gene has been up-regulated after four weeks of antiandrogen treatment (Knuuttila et al., 2018) and in the study 1 with ORX+ADX (I; Figure 5D).

5.3.4 Adrenalectomy combined with castration is more efficient than antiandrogen treatment alone

We compared the long-term effects of ORX+ADX to the ORX+antiandrogen Enza treatment in study 3 (II). To our interest the treatment response for ADX was more efficient than that of Enza, resulting to a slow tumor growth over the whole 6-weeks-long study period without no evidence for a treatment resistance. This resulted to a significantly smaller tumor volume and PSA concentration in ORX+ADX II group at the end of the study compared to that observed with Enza treatment. Furthermore, at the end of the study, the two of the 13 tumors did not express reliable amounts of PSA and were barely palpable. This result prompted us to further investigate the differences between key androgens (A-dione, T and DHT) and P_4 ORX+Enza and ORX+ADX treated tumors and mice in study 3 (II).

The results of our previous study 1 (I) showed us that the adrenals of ORX mice produce the androgens and P_4. In study 3 intratumoral P_4 was significantly higher than androgens, and the large drop in serum P_4 after ADX treatment again confirms that circulating P_4 in male mice is partially of adrenal origin (II; Figure 3B–C). Also, the high adrenal to serum ratio of steroids indicates the production of active androgens in the mouse adrenal (II; Figure 4A).

Enza treatment decreased intratumoral P_4 levels but did not affect adrenal P_4 production or serum levels in ORX+Enza treated mice (II; Figure 3A–C). This, together with the low tumor-to-serum ratio, indicates activation of P_4 metabolism in Enza-treated tumors, while in all other treatment groups the tumor-to-serum ratio was close to one, suggesting that P_4 metabolism in these tumors was minimal (II; Figure 4B). High intratumoral T and DHT concentration in Enza-resistant tumors indicates activation of intratumoral steroid synthesis using adrenal P_4 as a precursor. On the other hand, also the high production of A-dione, T and DHT in mouse adrenals after long-term Enza treatment potentially contributes to the higher concentration of DHT in tumors (II; Figure 3A–B).

5.4 Subcutaneous and orthotopic VCaP xenograft mouse models are comparable and have identical response to hormonal treatments

It is generally thought that *o.t.* tumor models are more clinically relevant than *s.c.* tumor models because of the organ-specific tumor microenvironment. It is known that both models exhibit important comparable properties such as androgen-dependent growth and circulating PSA. We, thus, compared *o.t.* and *s.c.* tumors for their similarities and differences between tumor growth, status of AR and steroids in intact, CRPC, and enzalutamide treated mice. However, based on this study, we can conclude that the *o.t.* and *s.c.* VCaP xenograft models resembled each other.

5.4.1 VCaP tumors have an identical growth pattern subcutaneously and orthotopically

Orthotopic and subcutaneous VCaP xenografts in ORX hosts in the study of Knuuttila et al., 2014 and studies 1 (I) and 3 (II) of this thesis, displayed the fundamental properties of CRPC. In both models ORX and antiandrogen treatment caused a transient tumor growth inhibition as measured by PSA and/or tumor size (Table 6). In both models, serum PSA was measurable two weeks after inoculation (I; Figure 1C) and castration caused a temporary decrease in PSA levels which started to increase to castration-resistant growth. Castrated and /or Enza treated *o.t.* mice were sacrificed earlier and had a higher PSA, than the corresponding *s.c.* tumors bearing mice, but the response to treatments was similar in both models. It should also be noted that, no metastasis to other organs was observed in either model in these experiments.

The expression response of AR to treatment is also similar in both VCaP models. Expression of full-length androgen receptor (AR-FL) and main splice variants AR-V1 and AR-V7 mRNA and AR protein levels were increased in *s.c.* VCaP tumors by ORX in study 1 (I) as well as in the *o.t.* model (Knuuttila et al., 2014, 2018). Also, similar growth properties were confirmed with *s.c.* VCaP xenografts in studies 1 (I) and 3 (II). Immunohistochemical staining revealed that the tumors express AR in protein level very similarly and its location in the nucleus was comparable in both models (Figure 7).

Table 6. Serum PSA (Mean ± SEM) levels measured at study endpoints and duration of tumor growth and enzalutamide treatment. PSA with Mean ± SEM (µg/l), ORX (orchiectomy), Enza (enzalutamide), *o.t.* (orthotopic) and *s.c.* (subcutaneous).

MODEL	PSA (µg/l) (MEAN±SEM)	DAY POST INOCULATION	DAY POST ORX	DAY POST ENZA	STUDY
INTACT *o.t.*	72,2 ±10,57	21-98	-	-	(Knuuttila et al., 2014)
INTACT *s.c.*	65,2 ±10,66	84	-	-	1 (I)
ORX *o.t.*	1.9 ± 0,24	14–35	7		(Knuuttila et al., 2014)
ORX I *s.c.*	2,47 ±0,52	42	10	-	3 (II)
ORX *o.t.*	53,39 ±7,61	66	28	-	(Knuuttila et al., 2018)
ORX *s.c.*	22,81 ±4,86	105	56	-	1 (I)
ORX II *s.c.*	14,7 ±3,33	112	76	-	3 (II)
ORX+ENZA *o.t.*	12,95 ± 1,75	77	49	7	(Knuuttila et al., 2018)
ORX+ENZA I *s.c.*	3,23 ±0,57	76	34	5	3 (II)
ORX+ENZA *o.t.*	35,74 ±6,72	91–133	70–98	28	(Knuuttila et al., 2018)
ORX+ENZA II *s.c*	17 ±3,00	116	76	47	3 (II)

Histologically, s.c. tumors were more homogeneous and consisted almost entirely of VCaP cell mass only, whereas o.t. tumors also contained murine prostate tissue (Figure 10).

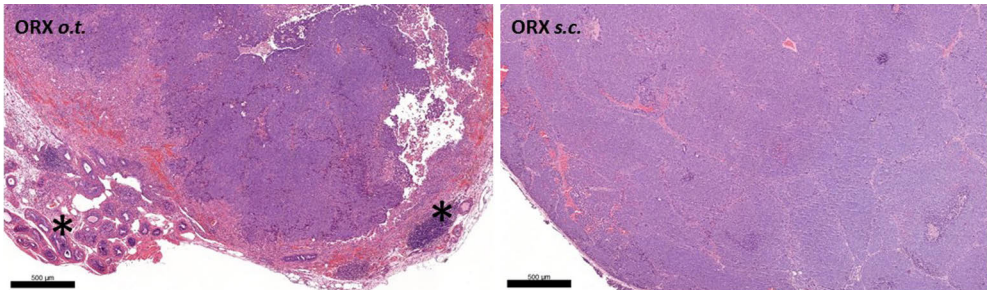

Figure 10. Representative HE stainings of orthotopic (*o.t.*) and subcutaneous (*s.c.*) ORX tumors. The staining shows mouse prostate tissue in the tumor marked as asterix in the figure. Scale bars = 500 µm.

5.4.2 Steroid levels in orthotopic and subcutaneous tumors are comparable during hormonal treatments

Detectable levels of P_4, A-dione, T and DHT were measurable in the tumors of intact and ORX (CRPC) mice in study by Knuuttila et al., 2014 as well as in studies of this thesis 1 (I) and 3 (II). Significant difference between *o.t.* and *s.c.* models was not observed in the concentrations of these steroids in intact or castrated tumors, although the durations of the treatments and the measurement time points varied (Figure 11). In intact mice with *o.t.* tumors, steroids were measured 21–98 days after inoculation and in intact mice with *s.c*. tumors, steroids were measured 84 days after inoculation. In ORX mice with *o.t*. tumors, steroids were measured 66 days after inoculation and 28 days after ORX and in ORX mice with *s.c.* tumors, steroids were measured 105 days after inoculation and 56 days after ORX. The concentration of A-dione and T decreased in the tumor as a result of ORX, but P_4 and DHT are at the same level as before castration. This, together with our previous results, supports the conclusion that in both models the tumor *de novo* DHT steroid synthesis precursor P_4, is likely of adrenal origin.

Also, the duration of the ORX effect does not seems to influence the tumor P_4, T or DHT concentrations in either model, but the A-dione concentration is higher in the *o.t*. model. However, A-dione is only an intermediate in the synthesis of T and DHT and has not been found to have a direct effect on tumor growth suggesting local androgen synthesis. Instead, with the longer Enza treatment, the antiandrogen effect is observed as an increase in T and DHT concentrations in Enza II tumors (Figure 12) probably due the resistance, but here again there is no difference in the antiandrogen effect itself between the models.

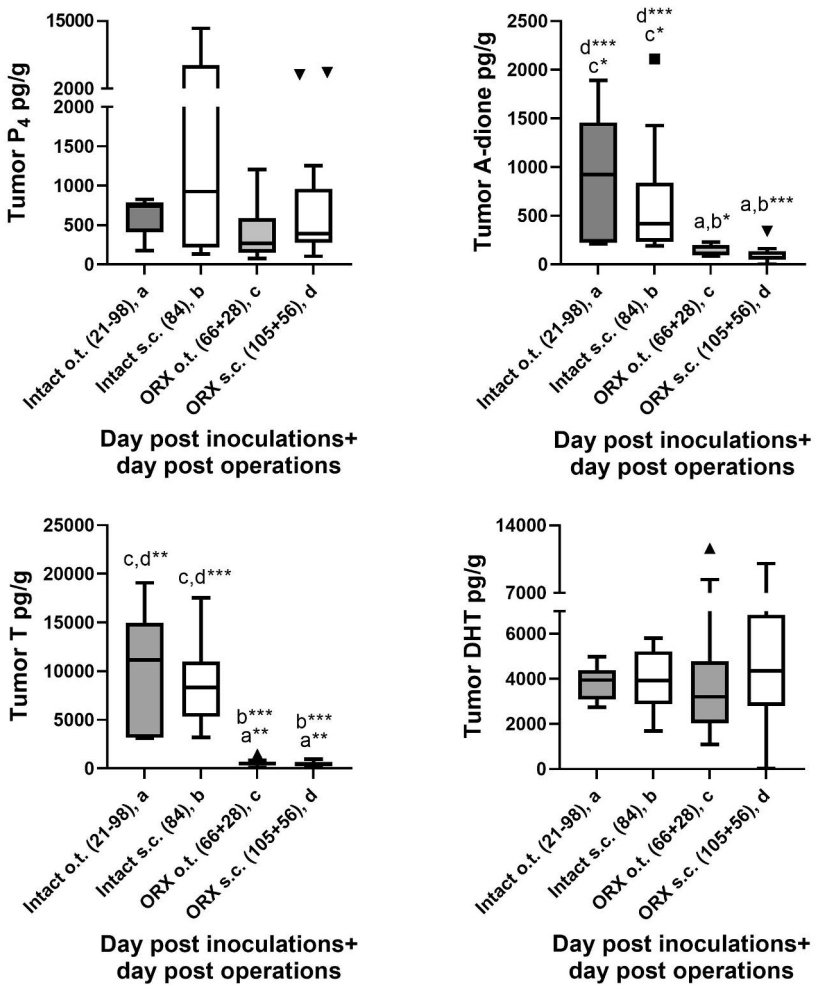

Figure 11. Concentrations of tumor progesterone (P_4), androstenedione (A-dione), testosterone (T) and dihydrotestosterone (DHT) in tumors in intact and orchiectomized (ORX) mice.

Groups are denoted as follows: **Intact o.t.** mice with orthotopic (*o.t.*) tumor n= 6, steroids measured 21–98 day post inoculation; **Intact s.c.** mice with subcutaneous (*s.c.*) tumor n= 10, steroids measured 84 day post inoculation; **ORX o.t.** (ORX mice with *o.t.* tumor) n= 15, steroids measured 66 day post inoculation and 28 day post ORX; **ORX s.c.** (ORX mice with **s.c.** tumor) n= 14, steroid measured 105 day post inoculation and 56 day post ORX.

Kruskal–Wallis with Dunn's post hoc tests was used for statistical analyses. Data are expressed as the median and range with Tukey box and whisker plots The columns of the different treatments are presented in alphabetical order and the significances are marked with asterisks after the letter. *P < 0.05, **P < 0.01, and ***P < 0.001. Orthotopic (*o.t.*) data are from study Knuuttila et al., 2014.

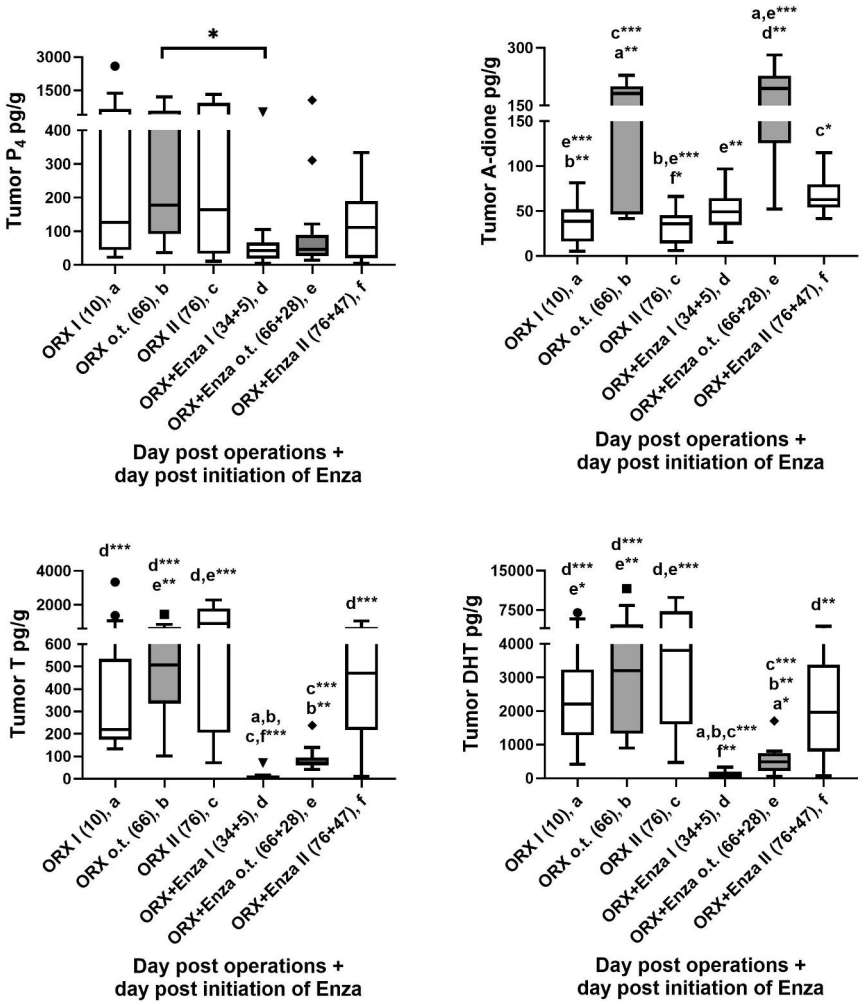

Figure 12. Concentrations of progesterone (P_4), androstenedione (A-dione), testosterone (T) and dihydrotestosterone (DHT) in tumors in orchiectomized (ORX) and orchiectomized and enzalutamide-treated (ORX+Enza) mice.

White bars depict *s.c.* tumors and gray *o.t.* tumors. Groups are denoted as follows: **ORX I** (ORX mice with *s.c.* tumors, sacrificed 10 days after ORX) n=17; **ORX *o.t.*** (ORX mice with *o.t.* tumors, sacrificed 66 days after ORX) n=15; **ORX II** (ORX mice with *s.c.* tumors, sacrificed 76 days after ORX) n=17; **ORX+Enza I** (*s.c.* tumors bearing mice sacrificed 34 days after ORX and 5 days after initiating Enza treatment) n=14; **ORX+Enza o.t.** (ORX mice with *o.t.* tumor, sacrificed 66 days after ORX and 28 days after initiating Enza) n=14; and **ORX+Enza II** (*s.c.* tumors bearing mice sacrificed 76 days after ORX and 47 days after initiating Enza treatment) n=14.

Kruskal–Wallis with Dunn's post hoc tests was used for statistical analyses. Data are expressed as the median and range with Tukey box and whisker plots. The columns of the different treatments are presented in alphabetical order and the significances are marked with asterisks after the letter. *P < 0.05, **P < 0.01, and ***P < 0.001. Orthotopic (*o.t.*) data are from study Knuuttila et al. 2018.

6 Discussion

Drug resistance is common in many diseases, including cancers, where it is a particularly urgent and unresolved issue. Resistance occurs in almost all types of cancer and against numerous different types of drugs with distinct mechanisms of action. In recent years, several studies have led to an improved prognosis of prostate cancer (PCa) patients, thanks to new therapies such as the second-generation androgen receptor (AR) antagonist, enzalutamide. Although these agents have shown increased overall survival (OS), some patients do not benefit from it at all or acquire resistance during treatment (Scher et al., 2012). The multifactorial mechanisms responsible for this phenomenon are related to various resistance mechanisms and the way cells adapt to prevailing condition. An example of this is the decrease in serum androgen levels caused by androgen deprivation therapy (ADT), as a result of which PCa cells are able to synthesize androgens themselves (Cai et al., 2011; Locke et al., 2008) and modify the AR so that even low androgen levels could activate it (Chen et al., 2004; Linja et al., 2001). This observation also supports that the mouse is a suitable model to study steroid metabolism and thus the results are more comparable to humans.

6.1 The VCaP xenograft model can be used to model the course of prostate cancer better than previously thought

Our research focused on the AR-positive PCa, because in most of the castration resistant prostate cancer (CRPC), indicated by the rising prostate specific antigen (PSA), AR signaling remains active in late stage of PCa (de Bono et al., 2011; Scher et al., 2012). Xenograft models generated with PCa cells are widely used (Kelland, 2004; Pretlow et al., 1993; Rygaard & Povlsen, 1982; van Weerden & Romijn, 2000) for studying PCa biology and drug responses. Vertebral prostate cancer (VCaP) xenografts from orchiectomy (ORX) mice have several key features of clinical CRPC, including castration-resistant growth, intratumoral androgen biosynthesis, significant expression of both full-length AR and various AR splice variants, and responsiveness to AR antagonists (Knuuttila et al., 2014; Knuuttila et al., 2018).

Our research also addressed a question of the role of mouse adrenals in production of steroids in VCaP xenograft model. The relevance of mouse CRPC models has been questioned due to species differences in extragonadal sex steroid production (Locke et al., 2008; Stuchbery et al., 2016). In humans, importance of weak androgens dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEA-S), produced by adrenal glands, are acknowledged as precursors for the intratumoral synthesis of testosterone (T) and dihydrotestosterone (DHT) in PCa (Evaul et al., 2010). In contrast, murine adrenal production of DHEA and DHEA-S has been considered insignificant, and thus, murine adrenals have not been thought to have a significant contribution to local androgen biosynthesis in CRPC xenografts (van Weerden et al., 1992). In our studies, we have challenged this conclusion, and by using up-to-date steroid profiling methodology, provided proof for the significance of murine adrenal steroids in regulation of CRPC xenograft growth.

The role of adrenal steroids has not been elucidated in previous VCaP xenograft studies, possibly due to the perception that mouse adrenal glands do not have the enzymatic machinery required for androgen production (van Weerden et al., 1992) and this species-difference has been considered a major drawback, as regards using the mouse as model for CRPC. Compared to previous immunoassay based steroid measurement methods, the latest MS-based gas chromatography and liquid chromatography-tandem mass spectrometry (GC-MS/MS, LC-MS/MS) applications allow more reliable and high accuracy measurement of steroid hormones from both body fluids and tissue homogenates picomolar or even femtomolar concentrations (Handelsman & Wartofsky, 2013; Knuuttila et al., 2019). Partly due to this more precise analytical method, our data clearly show a contribution of adrenal gland to the intratumoral steroids in the castration-resistant VCaP tumors and to their androgen-dependent growth, also evidenced by similar target gene responses after antiandrogen treatment and adrenalectomy (ADX). Taken together, ADX impairs VCaP tumor growth in ORX mice, indicating that adrenal steroids promote the growth of castration-resistant VCaP xenografts.

As we have shown, after ADX, the VCaP tumors slowly begin to grow again. The same phenomenon has been observed in humans (Bhanalaph et al., 1974). Although adrenalectomy or hypophysectomy has been shown to have transient palliative effects in patients who failed medical or surgical castration, these methods have not been widely used due to the complexity and the side effects of this approach (Denmeade & Isaacs, 2002). The data, thus, indicated that mouse is a valid preclinical model to study properties of CRPC and to test CRPC treatment strategies. We also showed that the tumor inhibitory effect of ADX was not abolished by treatment with the physiological dose of corticosterone. Mice tolerate ADX well, and do not require glucocorticoid replacement for their survival. Thus, we proved that the effect of ADX on VCaP xenograft growth is not explained by the lack of glucocorticoids or reduced general

well-being of the host. This is a great importance as it demonstrates similarity between rodents and humans in terms of the adrenal contribution for the castration-resistant growth of PCa, and thus, indicate that the data obtained from the mouse models likely translate to humans better than previously anticipated. This result may have an impact on several studies with xenografts in PCa.

However, it should be noted that the steroidogenic pathways in rodent and humans are different. The cytochrome P450 Family 17 Subfamily A Member 1 (CYP17A1) is a key enzyme of the steroidogenic pathway, which converts the precursors of cortisol biosynthesis 17-OH pregnenolone and 17-OH progesterone to the precursors of sex steroid biosynthesis DHEA and androstenedione (A-dione). In the rodent CYP17A1 is more active on 4-ene steroids pathway (17-OH progesterone and A-dione) and it has different substrate specificity (Fevold et al., 1989; Namiki et al., 1988) while in the human and primates, delta 5 pathway (17-OH pregnenolone and DHEA) is more active (Flück et al., 2003). This is probably why rodents produce mainly 4-androstenedione and corticosterone and not DHEA and cortisol like humans. Because of the lack of DHEA-S synthesis, rodents have been criticized for modeling human steroidogenesis. Although DHEA has been observed in mouse serum in some studies (Chubb & Desjardins, 1983; Tagawa et al., 2006), circulating DHEA levels are much lower in mice than in humans. However, there are also similarities between human and rodent DHEA steroid metabolism. For example, rodents are able to metabolize DHEA, as castrated rats given DHEA have a dramatic increase in prostate size and DHT levels (Labrie et al., 1988), and the same observation was recently made also with mice (Colldén et al., 2022).

It is well known that the microenvironment of the prostate stroma contains several components that are anatomically and physiologically important for normal glandular function as well as for the development and progression of PCa (Bahmad et al., 2021; Niu & Xia, 2009). In this thesis we have conducted VCaP research using a subcutaneous (*s.c.*) model. Contrary to expectations, in our earlier study with orthotopic (*o.t.*) VCaP tumors did not form metastases during the experiment (Knuuttila et al., 2014), even though the cells are originally derived from a vertebral metastasis (Korenchuk et al., 2001). Thus, the effect of steroid synthesis on the development of metastases is difficult to study with this model. However, the development of local VCaP metastases to lymph nodes has been observed to take an average of 12 weeks to develop (Linxweiler et al., 2018). This is a rather long time in a xenograft experiment, especially if treatments for metastases are to be studied. Based on the results of our studies, the *o.t.* VCaP tumor microenvironment does not appear to have an effect on tumor steroid concentrations compared to *s.c.* xenografts, although several exogenous factors, including cytokines, growth factors, and paracrine cell interactions, have been found to promote steroid production in PCa cell lines *in vitro* (Mostaghel, 2013).

6.2 The effects of castration and antiandrogen therapy are only transient

It is known that orchiectomy, estrogens, or gonadotropin-releasing hormone (GnRH) agonists or antagonists (via inhibition of LH secretion) reduce circulating testosterone by 90–95% (Labrie, Dupont, & Belanger, 1985; Labrie et al., 1980; Moghissi et al., 1984; Waxman et al., 1983). However, a much smaller effect, only 50–70% reduction, is seen in the concentration of DHT, which truly indicates the internal androgen synthesis in the tissue (Belanger et al., 1986; Labrie, Dupont, & Belanger, 1985). This is repeatable in the VCaP model and is in line with the outcome of our research results.

Enzalutamide has been approved for PCa patients who have non-metastatic castration-resistant prostate cancer (nmCRPC), metastatic hormone-sensitive prostate cancer (mHSPC) or metastatic hormone-sensitive prostate cancer (mCRPC). Enzalutamide improves the OS of CRPC patients after chemotherapy by several months in about 50% of the patients, but almost all patients develop resistance to treatment (Scher et al., 2012). The mechanisms associated with therapy resistance are still unsolved and this is a major concern. Our results are expected to provide a novel understanding of the mechanisms resulting in antiandrogen resistance, thus, being of high value for developing better drugs to treat patients with CRPC. So far, we have already shown that the antiandrogen resistance is associated with the re-activation of androgen synthesis in the tumors and, above all, an increase intratumoral DHT. Indeed, increased DHT level reduces AR inhibition by enzalutamide (Richards et al., 2012) and DHT has a higher affinity for AR than enzalutamide (Tran et al., 2009). Therefore, competition for enzalutamide binding to the AR may occur with increased androgen synthesis. Thus, our data suggest a novel treatment target for the action for antiandrogens, namely inhibiting the local steroid synthesis in the tumors such as the non-steroidal selective CYP11A1 inhibitor ODM-208, which inhibits the synthesis of all steroid hormones and their precursors (Karimaa et al., 2022).

Although the adaptation associated with hormonal therapy is mediated by activation of AR-dependent pathways, it can also be attributed to the crucial mechanisms of progression that bypass AR signaling. Endocrine therapies can induce therapeutic resistance through clonal selection and consequent growth of cell clones independent of AR signaling (Pisano et al., 2021; Roudier et al., 2003; Shah et al., 2004). In our studies, the sequencing results also suggested that the mechanism of resistance could be similar to other cancers, and according to direct markers of androgenic activity, pathways and processes such as inflammation, angiogenesis, invasiveness, epithelial-mesenchymal transition (EMT) and neuroendocrine development, are induced also to affect the progression of tumorigenesis.

Dividing of mechanisms into AR-dependent and independent ones is not entirely clear, as our results indicates with regulation of nephroblastoma overexpressed

(NOV) gene. Besides NOV is a marker of androgen action (Knuuttila et al., 2018; Wu et al., 2014) and acting as a tumor suppressor in PCa (Fong et al., 2017; Wu et al., 2014), it is an inhibitor of the P1K3/AKT/mTOR pathway (Huang et al., 2019). P1K3/AKT/mTOR pathway is also common in other cancers, where it regulates, e.g., cell survival, growth and proliferation, angiogenesis metabolisms, and differentiation of stem cell like properties (Bitting & Armstrong, 2013). The reduced expression of NOV in our study is consistent with increased cell proliferation in Enza-resistant tumors compared with Enza-responsive tumors, and thus downregulation of NOV could be one of the effects by which increased intratumoral DHT drives Enza resistance.

Likewise, Procollagen-Lysine,2-Oxoglutarate 5-Dioxygenase 2 (PLOD2) in our study was among the most up-regulated genes in the Enza-resistant tumors, has been shown to mediate resistance by promoting stemness through P1K3/AKT/mTOR pathway *in vitro* and *in vivo* in other cancers (Sheng et al., 2019; Song et al., 2017). Lysyl hydroxylase 2 (LH2, encoded by the PLOD2 gene) is the key enzyme mediating the formation of the stabilized collagen cross-links also needed for cancer cell migration and invasion (Du et al., 2017) and in many cancers, increased expression of PLOD2 is known to be an independent factor of poor prognosis and associated with reduced survival (Du et al., 2017).

According to studies conducted in recent years, the AR pathway remains active even after the resistance to new AR-targeted therapies enzalutamide and abiraterone (Li et al., 2013; Mostaghel et al., 2011) and these AR-targeted therapies usually maintain AR expression (Antonarakis et al., 2014; Joseph et al., 2013). Thus, even at this stage, AR is still a potential drug target. However, selective pressure by second-generation antiandrogens, increases the importance to use of more efficacy combined approaches against other signaling pathways such as PI3K/AKT/mTOR in enzalutamide-resistant PCa, but targeting steroidogenesis and AR action still remains as a viable treatment strategy in late stage of PCa. Although the introduction of enzalutamide as a second-line hormonal treatment in mCRPC patients has resulted in significantly better prognosis in patients, the mechanisms of the disease are still partially unknown. This led us also to study the whole-body steroid homeostasis and the interaction between the host and the tumor.

6.3 Adrenalectomy combined with castration is more effective than castration and antiandrogen treatment together

Androgen synthesis by the adrenal glands is known to continue despite medical or surgical castration (Hu et al., 2010) and the role of adrenal androgens as drivers for CRPC growth in humans is generally accepted. Increased AR expression contribute

to increasing AR responses to low levels of androgen, but on the other hand, it has also been shown that AR itself can modulate androgen synthesis in PCa (Audet-Walsh et al., 2017). Thus, it is noteworthy that AR is expressed in the adrenal cortex of both rodents (Bentvelsen et al., 1996; Sar et al., 1990) and humans (Rossi et al., 1998).

Surprisingly little is known about androgen action in adrenal cortex in preclinical animal models. In both humans and mice, the sex steroid-producing cells in adrenal cortex is thought to be generated during the fetal period in specific zones (Kim et al., 2009). In humans, this zone is known as the "fetal zone", and the mouse homologue is called the "X-zone" (Kim et al., 2009). In the mouse, these fetal cells of X-zone are maintained for some time after birth until their atrophy, but cells reappear in adult males after castration (Huang & Kang, 2019). However, this post-castration X-zone regeneration and AR expression in the mouse adrenal suggest that androgens and AR are important regulators of the adrenal cortex also in the mouse and may be one factor regulating mouse adrenal steroid synthesis after castration.

In line with previous results of Kero et al., 2000, we found that ORX caused a significant induction of adrenal luteinizing hormone (LH) receptor (*Lhcgr*) expression in mouse, and this expression had a strong correlation with the expression of *Cyp17a1*. ORX is known to increase circulating LH levels in mice (Lindzey et al., 1998) and humans (Hampl et al., 1988) and thereby inducing the expression of its receptor and promoting steroidogenesis in the adrenal cortex (Kero et al., 2000). Thus, the use of Gonadotropin-releasing hormone (GnRH) agonist instead of antagonists may be advantageous in the treatment of PCa patients, but the factors and mechanism of adrenal steroid synthesis should be further investigated.

Previous studies have reported increased circulating concentrations of progesterone (P_4) in mice after ORX (Locke et al., 2008; Nilsson et al., 2015) and speculated that its production originates from tumor cells (Dillard et al., 2008; Locke et al., 2009). In our study, ORX alone had no effect on intratumoral and serum P_4 or DHT levels, whereas ADX combined with ORX results in a marked decrease in P_4 and DHT steroids. This, together with the very high levels of P_4 in the adrenal gland and the decreased tumor/serum ratio after ORX+ADX, strongly indicates that P_4 originates from the adrenal gland and is likely precursor for the local androgen production in castration-resistant VCaP tumors.

The elevated AR levels in CRPC tumors (Yuan et al., 2014) may enable AR activation by low-affinity ligands such as P_4. However, P_4 is only a weak agonist for wild-type AR, being more than 200-fold weaker than DHT (Phillips et al., 1990). Thus, the current data suggest that DHT, present at significant concentrations in castration-resistant VCaP xenografts, is a major driver of intratumoral AR signaling in ORX and ORX+ADX mice. However, P_4 can act as a substrate for steroid 5α-reductase in a backdoor pathway for DHT synthesis that bypasses the need for T as

an intermediate (Andersson & Russell, 1990). This is possible due to the expression of 5α-reductase enzymes in VCaP xenografts grown in ORX+ADX mice and because intratumoral levels of P_4 and DHT are comparable to tumor levels in intact animals. These findings, together with a significant decrease in intratumoral T after ORX and a further decrease after ADX, support the conclusion that adrenal P_4 is a precursor of intratumoral DHT synthesis via the backdoor pathway. The intermediate steroids of backdoor pathway were also recently measured in human PCa tissue, and it was noticed that the increasing supply of precursors during CRPC may lead to the backdoor pathway producing the most potent androgens (Deb et al., 2021).

Consistent with clinical data (van der Kwast et al., 1991), our previous study confirmed that also in VCaP xenografts ORX induces the expression of full-length AR and its splice variants (Knuuttila et al., 2014), and that antiandrogen treatment further stimulates AR expression in castration-resistant VCaP tumors (Knuuttila et al., 2018). The effect of ADX is similar to antiandrogens as it appears to up-regulate the expression of AR mRNA and its splice variants and stimulate intratumoral DHT synthesis. At the protein level, however, we did not observe any induction of AR after ADX or enzalutamide treatments, likely because of lower stability of protein in the presence of only a low amount of the ligand (Lee & Chang, 2003; Symss et al., 1985).

6.4 Therapy predictive biomarkers are needed to plan targeted treatments for patients

PSA is by far the most widely used biomarker for PCa screening and monitoring during treatment (Pinsky et al., 2017). However, it does not tell how aggressive the cancer diagnosed is and its poor predictor of survival (Heller et al., 2018). There are currently no markers to distinguish between the different stages of PCa. An increasing number of different therapeutic options may prolong survival in mCRPC patients, and it would be necessary to find proper biomarkers that simultaneously guide optimal treatment decisions and predict which patients will benefit most from different treatments.

So far, only a few biomarkers that predict the therapeutic response to PCa are in use. Markers e.g., the AR splice variant, AR-V7, measured in the circulating tumor cells to predict resistance to the enzalutamide and abiraterone (Antonarakis et al., 2017; Armstrong, Halabi, et al., 2019) and mutation in the BRCA 1 and 2 genes from PCa tissue to predict response to poly (ADP-ribose) polymerase (PARP) inhibitor (de Bono et al., 2020), are tested already in clinical studies. It has also been clinically shown that patients with metastatic castration-sensitive PCa with the hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1) 1245A>C variant receiving ADT have a shorter time to develop CRPC and a shorter OS than

men with HSD3B1 1245A (Hearn et al., 2020). This HSD3B1 1245C variant encodes a stable 3β-Hydroxysteroid dehydrogenase (3β-HSD1) enzyme that leads to the conversion of adrenal DHEA to DHT in the tumor (Chang et al., 2013). Still new therapy predictive biomarkers would be needed due to the heterogeneity of PCa.

Androgens are involved in the development and progression of both PCa and benign prostate hyperplasia. Serum T level has been used as a prognostic factor for PCa being the main androgen in the circulation, while it's more active metabolite DHT is mainly present in the prostate gland (Ito & Horton, 1971). High serum testosterone levels before ADT predict the greater survival rate (Chodak et al., 1991) and low testosterone level during the ADT predicted longer response to ADT (Klotz, O'Callaghan, et al., 2015). Testosterone levels have also been studied to predict the efficacy of AR-targeted treatments. Patients with serum testosterone levels ≥ 5 ng / dl benefited more from AR-targeted treatment compared to patients with serum testosterone levels <5 ng / dl (Hashimoto et al., 2019). In another study, progression-free survival, and OS in men with higher serum testosterone levels were significantly worse than in men with lower serum testosterone levels when treated with docetaxel and cabazitaxel (Shiota et al., 2019).

Despite biological evidence for an association between T concentration and PCa, previous epidemiological studies have not found evidence of an association between androgen levels and the PCa development (Roddam et al., 2008). The impact of androgens on PCa carcinogenesis is still unclear and is difficult to study as prostate androgens do not correlate with serum levels (Cook et al., 2017; Marks et al., 2008) as we also demonstrated in our xenograft studies. Yet circulating concentrations of sex steroid hormones are likely to have poor accuracy to predict the intratumoral environment, as we have also observed in our studies. Consistent with this hypothesis, other studies have also shown the lack of correlation between intraprostatic DHT concentrations and circulating concentrations of T and DHT (Cook et al., 2017; Heracek et al., 2007; Miyoshi et al., 2014; Olsson et al., 2011). Considering these and our results, intraprostatic androgen levels, particularly DHT level, might be used in the future assessment and management of PCa.

Based on up-regulation of several well-characterized androgen-regulated genes, such as KLK3 (Kallikrein Related Peptidase 3), LOX (Lysyl Oxidase), ELL2 (Elongation Factor For RNA Polymerase II 2), FKBP5 (FKBP Prolyl Isomerase 5), TMPRSS2 (Transmembrane Serine Protease 2) and PMEPA (Prostate Transmembrane Protein, Androgen Induced 1), androgen effect was found to be greater in Enza-resistant tumors than in those collected during the Enza-responsive phase. The overall variation in the expression, among androgen-dependent genes, was significantly greater in Enza-resistant tumors than in Enza-responsive tumors, it indicates increased heterogeneity among tumors growing after long-term treatment with Enza. This increasing heterogeneity between tumors as cancer progresses poses

challenges for designing treatments. Thus, the transcriptomic signature could be used as a potential biomarker when tailoring therapies.

Nephroblastoma overexpressed (NOV, formerly CCN3) is a AR-related gene (Knuuttila et al., 2018; Wu et al., 2014) and has been shown to promote cell differentiation (Chen et al., 2017) and to display both tumor suppressor and tumorigenic role, depending on the cellular environment (Perbal, 2008). In this study, NOV was up-regulated after ORX+ADX, suggesting that ADX effect was similar to that shown for antiandrogens (Enza and apalutamide) in our earlier study (Knuuttila et al., 2018). However, with longer antiandrogen exposure, regulation of NOV expression was found to be down-regulated. Indeed, in recent studies NOV has defined as a tumor suppressor in PCa (Fong et al., 2017; Wu et al., 2014), and thus, its decreased expression is consistent with increased proliferation in Enza-resistant tumors compared with Enza-responsive tumors. In addition to being a direct target of ligand-activated AR (Wu et al., 2014), NOV is an inhibitor of the P1K3/AKT/mTOR pathway (Huang et al., 2019). Thus, inhibition of NOV may be one of the effects by which increased intratumoral DHT promote Enza resistance and could be used as a biomarker to predict the stage of this disease.

6.5 Future directions and limitations of the study

The heterogeneity of PCa signaling pathways has been proven by Genome-wide analyses, which have identified four major signaling pathways that are most frequently altered in PCa: i) the androgen receptor (AR); ii) the phosphoinositide 3-kinase (PI3K) pathway; iii) the Ras/Raf/MEK/ERK pathway; and iv) the retinoblastoma protein (pRB) signaling pathway (Georgi et al., 2014). Thus, combination therapy with inhibitors targeting different pathways may effectively delay development of resistant PCa, and therefore, further research is needed on how these targeted therapies should be used in combination with current established treatments, such as ADT.

Several mechanisms have been proposed to explain continuous AR signaling in a CRPC. The most important are the changes that allow increased adrenal androgen uptake to prostate tissue (Bosland, 2000; Chen et al., 2004; Labrie et al., 2005; Stanbrough et al., 2006; Nadiminty & Gao, 2012), *de novo intracellular* androgen synthesis (Locke et al., 2008; Montgomery et al., 2008; Cheng et al., 2010; Nadiminty & Gao, 2012), and down-regulation of steroid metabolism (Soronen et al., 2004) as well as amplification and overexpression of AR and its splice variants (Chen et al., 2004; Guo et al., 2009; Waltering et al., 2009; Hörnberg et al., 2011; Shiota et al., 2011). However, the mechanisms underlying androgenic ablation resistance are not yet fully understood and treatment options for CRPC are limited as shown. Although current treatment focuses on AR-dependent CRPC, there are

indications that the use of more effective androgen pathway inhibitors increases the incidence of AR-independent CRPCs, such as aggressive treatment-related neuroendocrine prostate cancer which has been observed 15-30% of patients after androgen ablation treatment (Aggarwal et al., 2018; Patel et al., 2019). Therefore, we need to identify predictive biomarkers that may help distinguish patients who will benefit from additional AR signaling-targeted therapy from those who does not or may become resistant to this treatment strategy.

Combination therapies that aim to inhibit AR-independent survival pathways and AR-negative cells as well as androgen-dependent cells could simultaneously delay the development of hormonal treatment resistance mechanisms. In this context, recent evidence has shown that for example the addition of an protein kinase B (AKT) inhibitor to abiraterone treatment showed superior antitumor activity than abiraterone alone, especially in patients with PTEN-loss (Phosphatase and Tensin homolog) tumors (de Bono et al., 2019) and PI3K/mTOR inhibitor plus enzalutamide in a phase I/II trial showed a statistically significant improvement in median serological and radiographic progression free survival compared to enzalutamide alone (Kolinsky et al., 2020; Sweeney et al., 2019).

There are limitations in this study that could be addressed in future research. First, the study focused only on VCaP xenograft model. One of the problems in PCa modeling is the small number of available characterized cell lines. Currently, only four cell lines (VCAP, LnCaP, 22Rv, MDA PCa2b) out of most used cell lines express AR and have ability to secrete PSA (Shi et al., 2019). Indeed, the VCaP cell line is known to be the only one that expresses high levels of only wild-type, non-mutated, AR and secretes PSA (Van Bokhoven et al., 2003). In addition, it responds well to androgen ablation and has classic features of CRPC, as we have shown in this study and previously (Knuuttila et al., 2014; Knuuttila et al., 2018). However, it is also noteworthy that the VCaP cell line has been shown to model PCa well, as it has also been used in *in vitro* and *in vivo* studies in the development of the clinically used enzalutamide and darolutamide (Moilanen et al., 2015; Tran et al., 2009).

Another limitation is that, although adrenalectomy combined with castration resulted in decreased intratumoral androgens and a reduction in tumor growth, it is difficult to translate this method to the patient. Even though adrenalectomy after castration has been shown to have transient palliative effects in patients, this method has not been widely used due to the complexity and side effects of this approach (Denmeade & Isaacs, 2002). However, surgical approach can be replaced e.g., by a recently developed non-steroidal selective CYP11A1 inhibitor ODM-208, that blocks the synthesis of all steroid hormones and their precursors that can stimulate the AR signaling pathway (Karimaa et al., 2022). This compound has been assessed in the CYPIDES (NCT03436485) phase II clinical trial where adrenal function is verified with hormone replacement therapy during ODM-208 treatment.

The VCaP ORX+ADX xenograft is a good preclinical model for example, studying combination therapies. Indeed, many combinations in clinical trials have already been performed with enzalutamide and abiraterone, but unfortunately, they did not show any benefit with this combination (Efstathiou et al., 2020; Morris et al., 2019). This is likely due the fact that enzalutamide is a potent inducer of cytochrome P450 family 3 subfamily A member 4 (CYP3A4) (Gibbons et al., 2015; Narayanan et al., 2016) and the same CYP3A4 is the major metabolic pathway for abiraterone (Bernard et al., 2015). Since enzalutamide stimulates the degradation of abiraterone, it is recommended to avoid CYP3A4 inducers when therapeutic option is available to minimize the risk of treatment failure (Bernard et al., 2015). On the other hand, the combination is likely to have been studied in a very heterogeneous group of patients for whom androgen levels have not been further defined prior to treatments. Therefore, it would be clinically important to identify those CRPC tumors with high intratumoral DHT levels in antiandrogen resistance and to personalize the combination of an antiandrogen and a steroid synthesis inhibitor for these patients to achieve intensive androgen inhibition and deprivation.

7 Conclusions

The objective of this thesis was to outline the relevant AR functions that contribute to CRPC growth, and to explore the mechanisms of resistance to anti-androgens. According to our hypothesis, androgen-dependent growth plays an active role also in the late stages of PCa.

In contrast to human, murine adrenals do not produce large quantities of DHEA and especially its sulphate (DHEA-S), and thus, the value of preclinical mouse models for studying androgen-dependency of CRPC has been questioned. In the study, we defined the role of murine adrenal steroids in CRPC growth. Mouse adrenal glands produce steroids and their precursors for intratumoral androgen synthesis, and thus mouse seem to be a better model for human PCa than previously expected. Furthermore, no significant differences were obtained in the tumor behavior when inoculated *s.c.* and *o.t.*

With this model we showed that, like the clinical CRPC, the castration-resistant VCaP tumors respond to Enza treatment. Like many CRPC patients, the Enza response in castration-resistant VCaP tumors is transient, and the treatment resistance in our preclinical model is associated with increased intratumoral T and DHT concentration, presumably via increased intratumoral synthesis of adrenal gland precursors.

Based on the results of this thesis, the following conclusions can be made:

1) Subcutaneous and orthotopic PCa VCaP xenograft mouse models have a similar response to hormonal treatments and have similar growth progression.
2) Antiandrogen treated CRPC VCaP xenograft model presents us with features similar to clinical CRPC.
3) The growth of CRPC VCaP tumors is partially adrenal driven.
4) Long exposure antiandrogen treatment leads to therapy resistance.
5) Tumor growth and endocrine treatment response are associated with high intratumoral DHT concentrations.

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I hope this work will stimulate further interest in finding new diagnostic and treatment possibilities for this disease to increase the health and survival rates of patients, particularly for those suffering from metastatic prostate cancer.

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