Targeting Therapy Resistance in Advanced Prostate Cancer

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Preface

During the work performed in this thesis, large-scale next-generation sequencing studies have increased our understanding of genomic and pathway alterations in prostate cancer. In particular, there have been large developments in understanding aberrations and mechanisms associated with resistance towards treatments targeting the androgen receptor signaling axis in advanced prostate cancer. Many articles published during this work are cited in the introduction to update the reader on recent clinical and genomic developments in the field and give a background for my interpretation and discussion of the results.

Many publications append raw or normalized transcriptome data that is used in the introduction and discussion within this thesis. In these cases, the original publications are generally cited, even if the authors have not explicitly reported the transcriptomic features within their publications.

All illustrations in this thesis were produced by the candidate. Where illustrations were in part based on published literature, this is noted in the legends.

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Multiple collaborators from many institutions have contributed to the work presented in this thesis, and are appropriately acknowledged in the appended publications and manuscript. Most notably, I use this occasion to thank my fellow group members Helene H. Grytli and Ingrid J. Guldvik for their scientific contributions and moral support. I also thank the scientific and social environment at the Department of Tumor Biology, headed by Gunhild M. Mælandsmo, for supplying the needed workspace.

I thank my friends and family for their interest in my work and understanding of my frequent absence.

To my loving wife and our beautiful daughters: Thank you for your love, patience, understanding, and support. You are my leading motivation.

Oslo, August 2019

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1. Abbreviations

AC	Adenylate Cyclase
ACLY	ATP Citrate Lyase
ACTH	Adrenocorticotropic hormone
ADRB	β-adrenergic receptor
ADRB2	β_2-adrenergic receptor
ADT	Androgen deprivation therapy
AI-CRPC	Androgen-indifferent CRPC
AR	Androgen receptor
ARBS	Androgen receptor binding site
ARE	Androgen response element
ARPI	Androgen receptor pathway inhibitor
AR-V	AR variant
AURKA	Aurora kinase A
BAD	Bcl-2-Associated Death Promoter
BCR	Biochemical recurrence
cAMP	Cyclic Adenosine Monophosphate
CD24	CD24 Molecule
CD31	Platelet and Endothelial Cell Adhesion Molecule 1
CHGA	Chromogranin A
ChIP-seq	Chromatin immunoprecipitation sequencing
c-Myc	MYC Proto-Oncogene, BHLH Transcription Factor
CRE	cAMP response elements
CREB	cAMP-responsive binding protein
CRPC	Castration-resistant prostate cancer
CSS	Charcoal-stripped fetal bovine serum
CTCF	CCCTC-Binding Factor
CYP17A	Cytochrome P450, family 17, subfamily A
DHEA-S	Dehydroepiandrosterone sulphate
DHT	Dihydrotestosterone
DRE	Digital rectal examination
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ENO2	Neuron-Specific Enolase II (NSE)
ENZ	Enzalutamide
EPAC	Guanine Nucleotide Exchange Protein
EPE	Extraprostatic extension
ERBT	External-beam radiation therapy
ETV1	ETS Variant 1
EAU	European Association of Urology
EZH2	Enhancer of Zeste homolog 2
FASN	Fatty Acid Synthase
FDR	False discovery rate
FOXA1	Forkhead Box A1
GDP	Guanosine Diphosphate
GPCR	G-protein coupled receptor
GR	Glucocorticoid Receptor
GS	Gleason score
GSEA	Gene set enrichment analysis
GSK3β	Glycogen Synthase Kinase β
GTP	Guanosine Triphosphate
H3K27me3	Trimethylation at histone 3 lysine 27
HER2	Human Epidermal Growth Factor Receptor 2
HMGCR	HMG CoA Reductase
HNPC	Hormone-naïve prostate cancer
HSP90	Heat Shock Protein 90
IGF-1	Insulin-Like Growth Factor

IL-6	Interleukin 6
JAG1	Jagged Canonical Notch Ligand 1
JAK	Janus Kinase
KGF	Keratinocyte Growth Factor
KLHL1	Kelch Like Family Member 1
LBD	Ligand-Binding Domain
LH	Lutenizing Hormone
LHRH	Luteinizing Hormone-Releasing Hormone
MAOA	Monoamine Oxidase A
MAPK	Mitogen-Activated Protein Kinase
mHNPC	Metastatic hormone-naïve prostate cancer
NAV1	Neuron Navigator 1
NE	Neuroendocrine
NED	Neuroendocrine differentiation
NEPC	Neuroendocrine prostate cancer
NEtD	Neuroendocrine transdifferentiation
NGF	Nerve Growth Factor
NKX3-1	Homeobox Protein Nkx3.1
NSE	Neuron-Specific Enolase II (ENO2)
NSG	Non-Obese diabetic severe combined immunodeficiency
Oct4	POU Class 5 Homeobox 1 (POU5F1)
OS	Overall survival
PAK4	p21-Activated Kinase 4
PCa	Prostate cancer
PCSM	Prostate cancer-specific survival
PDE4D7	Phosphodiesterase-4D7
PDX	Patient-derived xenograft
PFS	Progression-free survival
PI3K/AKT	Phosphoinositide 3-Kinase/Protein Kinase B
PKA	cAMP-Dependent Protein Kinase
PNI	Perineural invasion
PRC2	Polycomb Repressive Complex 2
PSA	Prostate-Specific Antigen (KLK3)
PSAn	PSA nadir
PTEN	Phosphatase and Tensin Homolog
RB1	RB Transcriptional Repressor 1
RCT	Randomized clinical trial
REST	RE1 Silencing Transcription Factor
RhoA	Ras Homolog Family Member A
RMA	Robust multi-array average
RNA-seq	RNA sequencing
ROCK	Rho-Associated Coiled-Coil Kinase
RP	Radical prostatectomy
RT	Radiation therapy
SCCP	Small-cell carcinoma of the prostate
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SLC6A2	Solute Carrier Family 6 Member 2
SLITRK3	SLIT and NTRK Like Family Member 3
SNP	Short nucleotide polymorphism
SNS	Sympathetic nervous system
SOX2	SRY-Box Transcription Factor 2
SOX9	Transcription Factor Sox 9
SRD5A	Steroid 5α Reductase
sRT	Salvage radiation therapy
StAR	Steroidogenic Acute Regulatory Protein
STAT	Signal Transducers and Activators of Transcription
SVI	Seminal vesicle invasion
SYP	Synaptophysin
T3	Triiodothyronine
TF	Transcription factor

TH	Tyrosine Hydroxylase
TMA	Tissue microarray
TME	Tumor microenvironment
TMPRSS2:ERG	Transmembrane Protease, Serine 2:ETS-related gene fusion
t-NEPC	Treatment-related neuroendocrine prostate cancer
TP53	Tumor Protein P53
TRUS	Transrectal ultrasound
TS	Tumor suppressor
TSG	Tumor suppressor gene
TUBB3	Tubulin Beta-3 Class III
TUR-P	Transurethral resection of the prostate
UDPGA	UDP-glucuronic acid
UGT2B	UDP-Glucuronosyltransferase
VEGF	Vascular Endothelial Growth Factor
β-agonist	β-adrenergic receptor agonist
βARK	β-adrenergic receptor kinase
βarr	β-arrestin

2. List of publications

Published articles and manuscripts in preparation included in the thesis

I. **Braadland PR***, Grytli HH*, Ramberg H*, Katz B, Kellman R, Gauthier-Landry L, Fazli L, Krobert KA, Wang W, Levy FO, Bjartell A, Berge V, Rennie PS, Mellgren G, Mælandsmo GM, Svindland A, Barbier O, Taskén KA: Low β_2-adrenergic receptor level may promote development of castration resistant prostate cancer and altered steroid metabolism. *Oncotarget* **2016**; 7:1878-1894.

* These authors contributed equally to this work

II. **Braadland PR**, Ramberg H, Grytli HH, Urbanucci A, Nielsen HK, Guldvik IJ, Engedal A, Ketola K, Wang W, Svindland A, Mills IG, Bjartell A, Taskén KA: The β_2-adrenergic receptor is a molecular switch for neuroendocrine transdifferentiation of prostate cancer cells. *Mol Can Res* **2019**; doi: 10.1158/1541-7786.MCR-18-0605 (*Epub ahead of print*).

III. **Braadland PR**, Sivanesan S, Grytli HH, Ramberg H, Guldvik IJ, Katz B, Gemma, Berge V, Taskén KA: Bicalutamide treatment in hormone-naïve prostate cancer associates with cross-resistance to androgen deprivation therapy (*Manuscript*).

Other contributions during the PhD period not included in the thesis

I. **Braadland PR**, Ramberg H, Grytli HH, Taskén KA: β-adrenergic signaling in prostate cancer. *Front. Oncol* **2015**; https://doi.org/10.3389/fonc.2014.00375.

II. **Braadland PR**, Giskeodegard G, Sandsmark E, Bertilsson H, Euceda LR, Hansen AF, Guldvik IJ, Selnæs KM, Grytli HH, Katz B, Svindland A, Bathen TF, Eri LM, Nygård S, Berge V, Taskén KA, Tessem MB: Ex vivo metabolic fingerprinting identifies biomarkers predictive of prostate cancer recurrence following radical prostatectomy. *Br J Cancer* **2017**; 117:1656-1664.

III. **Braadland PR**, Urbanucci A: Chromatin Reprogramming as an adaptation mechanism in advanced prostate cancer. *Endocr Relat Cancer* **2019**; 26(4): R211-R236.

3. Introduction

The majority of prostate cancers have favorable prognoses, but the disease remains a major cause of cancer-related deaths among men in Norway and the Western world. Locally advanced and metastatic prostate cancers are associated with poor outcomes and have a considerable negative impact on the quality of life of patients and their relatives, as well as the health care systems. Considerable efforts have been laid into the development of treatment options for men with advanced and/or metastatic prostate cancer, but resistance nearly invariably develops. Treatment-resistant prostate cancers show more aggressive clinical behavior and have often acquired a set of skills to combat drugs existing within the current treatment toolbox.

The vast majority of men who succumb to prostate cancer have been treated with one or more therapies targeting the androgen receptor (AR) signaling axis. Thus, resistance to these treatments is a major cause of death from prostate cancer. The overall aim of this thesis was to elucidate mechanisms responsible for resistance towards treatments targeting the androgen receptor signaling axis. With this knowledge, one may not only identify actionable targets for therapy-resistant tumors but also prevent resistance from developing (Figure 3.1). Finally, identifying patients likely and unlikely to respond to a given treatment will help to tailor disease management for each patient individually while preventing overtreatment.

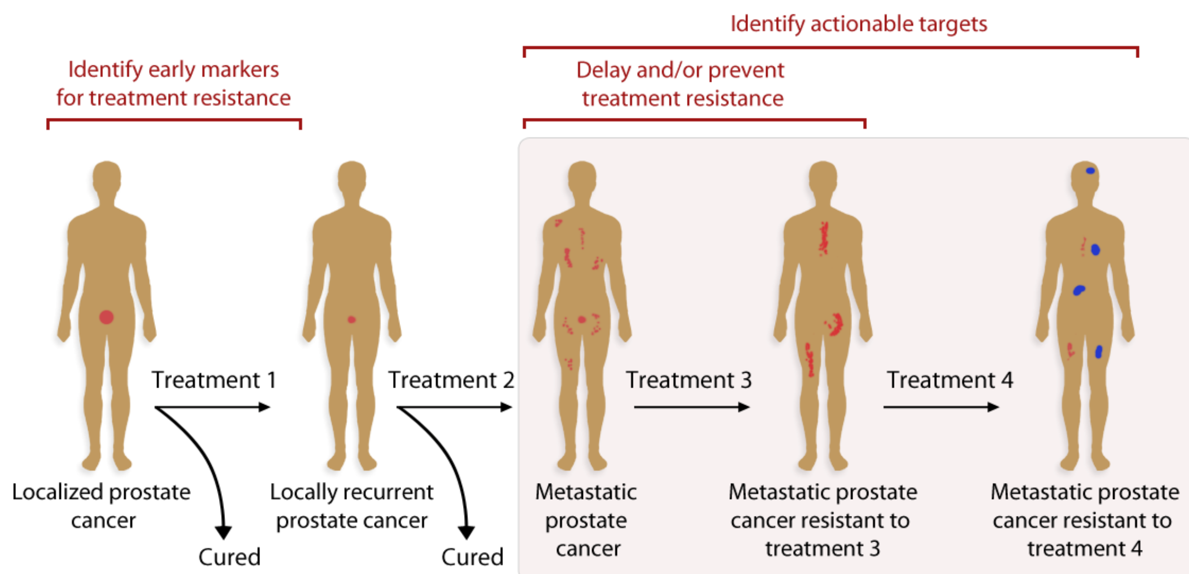


*Figure 3.1. **Schematic overview of the evolution of a clinically aggressive prostate cancer** that repeatedly recurs and becomes resistant to available treatments. The overall aims of the work in this thesis are indicated at the various disease stages in red.*

3.1. The healthy prostate gland

The prostate is an exocrine (secretory) gland in the male reproductive system. The prostate is situated below the urinary bladder, where it surrounds the urethra and connects it with the ejaculatory ducts running from the seminal vesicles (Figure 3.2A). The main structural components of the prostate are the glandular secretory ducts, which are formed by terminally differentiated columnar-shaped, secretory epithelial (luminal) cells and neuroendocrine (NE) cells lining the lumen (Figure 3.2B). Basal cells surround the secretory epithelium and are supported by a basal membrane and stromal cells. The luminal (exocrine) cells secrete a prostatic fluid into the prostatic lumen which intermixes with testes-derived spermatozoa-containing seminal fluid during ejaculation. Basal cells are critical for maintaining luminal cell differentiation and integrity of epithelial secretory ducts [1] but are not known to have secretory functions. NE cells are terminally differentiated, make out around 1% of the prostatic epithelium, and are believed to exert both neuronal and paracrine actions on adjacent cells [2].

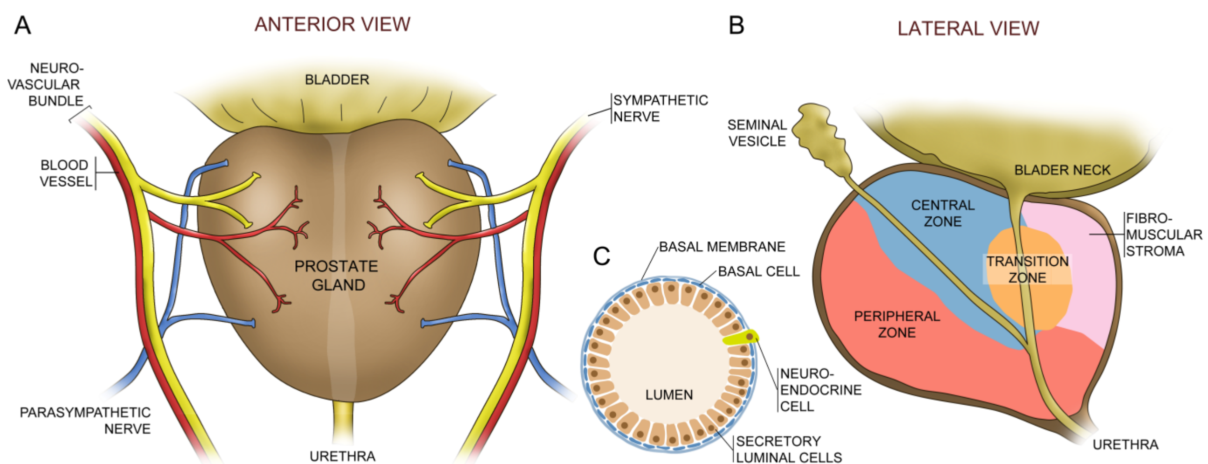


Figure 3.2 ***Anatomy of the prostate. A.*** *Anterior schematic view of the prostate gland. The prostate gland is situated below the bladder where it surrounds the urethra. The two neurovascular bundles posterolateral to the prostate consist of blood vessels and sympathetic nerve fibers which extend into the prostate. The sympathetic nerves predominantly innervate the base of the prostate, whereas parasympathetic nerves uniformly innervate the base and apex.* ***B.*** *Prostate zones. The fibromuscular stroma dominates the anterior prostate. The peripheral and central zones are situated in the posterior, with the peripheral zone extending laterally and anteriorly, and the central zone stretching mainly along the base. The transition zone is located centrally. The seminal vesicles reside posterior to the prostate, and ejaculatory ducts connect the urethra centrally in the prostate.* ***C.*** *A healthy glandular structure consisting of secretory luminal cells lining the lumen, with interspersed neuroendocrine cells. The luminal cells are supported by basal cells surrounded by a basal membrane, which separates the glands from surrounding stroma. References: [1-6].*

Anatomically, the prostate can be divided into three zones; namely the peripheral (located anterior, lateral and posterior), transitory (centrally) and central (anterior towards the base) zone (Figure 3.2) [4]. Fibromuscular stroma is also present in the anterior part of the prostate. The prostate is a highly innervated organ [3], and autonomic nerves play critical roles in prostate embryogenesis, pubertal maturation and differentiation [7], aside from their function on smooth muscle cells during ejaculation [8]. Whereas postganglionic parasympathetic nerves uniformly spread across the prostate, sympathetic postganglionic (adrenergic) nerves are more enriched in the base than the apex of the prostate [5, 6]. The sympathetic nerves path alongside blood vessels in the neurovascular bundles running posterolateral to the prostate and are connected to the CNS via acetylcholine-producing preganglionic nerve fibers.

3.2. Prostate cancer

In this section, the epidemiology of prostate cancer is briefly discussed, followed by an overview of prostate cancer oncogenesis, progression and heterogeneity.

3.2.1. Epidemiology

Each year, more than 5,100 men are diagnosed with prostate cancer in Norway [9]. With over 1000 deaths, prostate cancer represents the second leading cause of cancer-related death in Norway, only trailing lung cancer. Prostate cancer remains the most commonly diagnosed non-cutaneous cancer in European men [10]. The risk progressively increases with age and the number of first-degree relatives with prostate cancer [11], and around 9% of all cases are believed to have a hereditary component [10]. There are substantial differences in incidence, aggressiveness, and mortality between ethnic populations worldwide [12-14]. These differences largely diminish when men from low-risk countries migrate to high-risk countries, suggesting that behavioral factors (e.g. diet, sexual behavior, and metabolic syndrome) contribute to the overall risk [12, 15, 16].

3.2.2. Prostate cancer oncogenesis and progression

Prostate cancers primarily occur within the nerve-rich regions of the peripheral zone [6] and are generally characterized histologically by loss of basal cells and expansion of proliferative luminal cells (Figure 3.3). Hence, the majority of prostate cancers are of a luminal lineage [17]. Upon progression of a pre-neoplastic lesion, atypical cells may acquire invasive potential: The malignant cells increasingly proliferate and invade the basal membrane which leads to disruption of the benign morphological architecture. At this stage, the lesion is defined histologically as an adenocarcinoma (Figure 3.3). The tumor cells may extend their growth through and beyond the prostate capsule (extraprostatic extension, EPE), locally invade the seminal vesicles (seminal vesicle invasion, SVI) or spread to regional lymph nodes and distant organs.

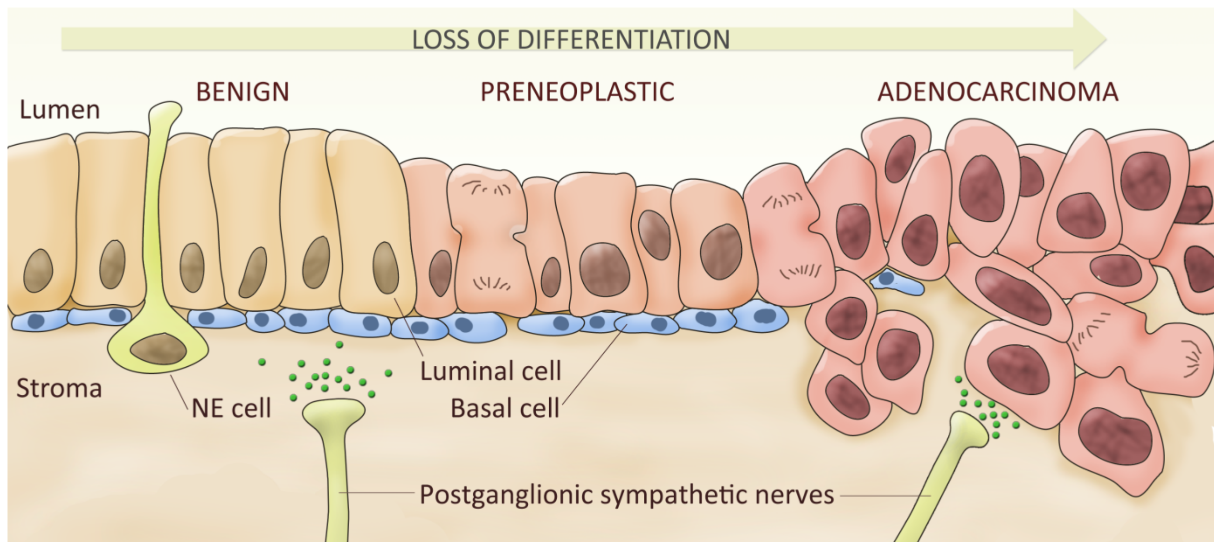


Figure 3.3. ***Loss of luminal differentiation during prostate cancer oncogenesis.*** *In the benign prostate histology, luminal cells, with occasional intermixed neuroendocrine cells, line the lumen. Postganglionic sympathetic nerves are in close contact with the stroma. The luminal cells are supported by basal cells, which again are supported by stroma. During oncogenesis, a pre-neoplastic stage (such as low- or high-grade prostatic intraepithelial neoplasia) may arise, characterized by some loss of luminal identity and atypical growth. In adenocarcinoma, the basal cell membrane and basal cells are lost, and malignant cells extend their growth into the lumen and the stroma, and may be in close contact with sympathetic nerves innervating the tumor microenvironment.*

3.2.3. Prostate cancer differentiation

Cellular differentiation relates to the process in which a cell loses pluripotency while gaining lineage-specific characteristics [18]. In humans, cellular differentiation predominantly occurs during embryogenesis, in which stem cells gradually narrow down their potential to reprogram to different cell lineages. This process is tightly orchestrated by gradual modifications in the DNA or chromatin structure which silences some genes while turning on transcription of other genes [18]. Once a cell has fully differentiated in its destined tissue, such as a luminal prostatic cell, dedifferentiation (increasing pluripotency) or direct transdifferentiation (bypassing an intermediate pluripotent state) rarely occurs. In cancer, including prostate cancer, transformed cells may hijack these processes. This enables the cells to dedifferentiate or alternate between different lineages through increased plasticity. The increasing loss of cellular differentiation associates with loss of normal cellular function and healthy tissue architecture, as can be assessed histologically. Alterations in the genome and epigenome have been shown to associate with increasing aggressiveness, loss of luminal cell identity, transdifferentiation, and poor clinical outcomes [19, 20].

3.2.4. Prostate cancer metastasis

The strong association between the development of distant metastases (i.e. spread beyond the pelvic area) and overall survival (OS) among prostate cancer patients has made distant metastatic spread a reasonable surrogate endpoint for OS [21]. A large autopsy study reported that the most common prostate cancer metastatic sites were bone (90%), lung (46%) and liver (25%) [22]. Alterations in the metastatic tropism is believed to change with more widespread use of existing and novel life-extending drugs for patients with late-stage systemic disease, however [23, 24].

Distant metastases are believed to primarily spread by hematogenous routes [22]. Perineural invasion (PNI), a phenomenon by which cancer cells invade and track nerve fibers [25], has been retrospectively associated with bone metastasis independently of other risk factors [26]. Thus, PNI may offer a route for cells to metastasize. Lymphatic spread represents a major regional metastatic route, exemplified by the prevalence of regional lymph node metastases even among patients with intermediate-risk, localized disease [27]. As mentioned, contiguous spread may also occur as EPE with or without seminal vesicle involvement partly depending on where in the prostate the tumor is situated.

3.2.5. Heterogeneity of prostate cancer

Localized, primary prostate cancers often consist of multiple lesions (i.e. "multifocal"). These lesions may display both intrafocal and interfocal histological and genetic heterogeneity [11, 28-30]. Intertumoral heterogeneity is also seen histologically [30-32] and genetically [11, 29], the latter being highly dependent on disease stage and prior treatment [33, 34]. Specifically, at the genomic level, intertumoral heterogeneity is observed, as the Cancer Genome Atlas Program (TCGA) Network identified mutually exclusive alterations involving gene fusions or mutations in 75% of primary tumors [35]. It is debated whether common alterations found in metastases and the primary tumor are clonally derived from the primary tumor or whether they occur subclonally as a result of treatment challenge [36]. Prostate cancer metastatic seeding has been shown to occur both monoclonally and polyclonally [36, 37]. In monoclonal metastatic spread, cells of monoclonal origin spread from the primary tumor to form a metastasis. Polyclonal metastasis refers to when multiple cells of different lineage spread to form a polyclonal metastasis. Interestingly, the clone(s) forming a prostate cancer metastasis may seed from foci with low-grade cancer, indicating that subclonal spread is not restricted to occur from the index tumor [38].

Basal cells have also been shown to be capable of cancer initiation in mouse models [17]. About every third prostate cancer tumor displays a basal-like gene signature with low expression of luminal

lineage markers [39], but the vast majority of tumors express markers associated with luminal differentiation and are determined epithelial adenocarcinomas by histopathology.

3.2.6. The androgen signaling axis

The basis of prostate cancer diagnostics, treatment, progression, and therapy resistance rely largely on the action of male sex hormones (androgens). Androgens are critical for the development, maturation, and differentiation of the healthy prostate, but also play pivotal roles in prostate cancer oncogenesis and progression [40]. There is a remarkable dependence of prostate cancer cells to androgens and AR signaling, which is exploited in the management of prostate cancer. This topic is therefore briefly introduced in the following section and more thoroughly described later.

Endogenous androgen production and secretion

Testosterone, which along with dihydrotestosterone (DHT) represent the two major androgens, is primarily produced in Leydig cells in the testes [41, 42] from which it is secreted into circulation to promote male characteristics. Around 40-50% of the testosterone in the prostate is not of testicular origin, however, but is converted from adrenal-derived precursor metabolites such as dehydroepiandrosterone sulphate (DHEA-S) in the prostate [41].

The hypothalamic-pituitary axis controls the endocrine secretion of androgens. Specifically, luteinizing hormone-releasing hormone (LHRH) regulates the secretion of pituitary luteinizing hormone (LH) which promotes testosterone secretion from Leydig cells. The adrenal androgens are produced upon adrenocorticotropic hormone (ACTH) which is under corticotropin releasing hormone (CRH) control.

Androgen action on the androgen receptor in the prostate

The canonical role of androgens is to bind to the AR, which is highly expressed in the secretory luminal cells in the healthy prostate and in prostate cancer cells of luminal lineage. AR stimulation in benign epithelial cells has been shown to lead to irreversible G0 growth arrest and luminal lineage commitment, exemplified by prostate-specific antigen (PSA, translated from the *KLK3* gene) secretion [43].

In absence of ligand, heat shock proteins form a complex with the AR, retaining it in the cytoplasm [40]. Androgens readily diffuse across cell plasma membranes due to their lipogenic molecular structure. Upon cytoplasmic binding to androgen or other non-androgenic ligands, the AR is phosphorylated and disassociated from the heat shock proteins. The AR then dimerizes and translocates to the nucleus where it can alter gene transcription by direct DNA binding or through

coactivating or corepressing functions toward other transcription factors (TFs) [40, 42, 44]. Non-genomic actions of AR have also been reported in prostate cancer (reviewed in [45]).

AR target genes, such as the canonical AR target gene *KLK3* [46], predominantly contain androgen response elements (AREs) in their proximal regulatory domain and are recognized by active AR proteins [47]. As transcription of these genes may increase upon androgen binding to the AR, they are termed androgen-responsive. Androgen-responsive genes commonly code for proteins involved in lipid and steroid hormone biosynthesis, cell cycle and DNA synthesis, collectively promoting growth, survival and luminal differentiation [40, 48]. The landscape of androgen-regulated genes in prostate cancer is contextual, as the gene regulatory networks (i.e. the AR cistrome) vary during disease progression [49]. As more thoroughly described later, this may occur through e.g. AR mutations, truncated AR variants, epigenetic alterations, regulation of expression of co-activators and repressors and other TFs acting in symphony with the AR, and are dependent on prior treatment regimens and androgen dependence [40, 48, 50].

3.3. Diagnosis and staging

The introduction of the blood-based PSA test revolutionized the diagnostic landscape of prostate cancer. Since the AR is expressed and active both in benign and malignant luminal cells, PSA is prostate-specific and not cancer-specific *per se.* However, an elevated PSA typically leads to suspicion of a cancer diagnosis, inquiring follow-up diagnostic tests such as digital rectal examination (DRE), transrectal ultrasound (TRUS), biopsies and imaging modalities.

Only a biopsy test can establish a prostate cancer diagnosis. The presence of atypical growth morphologies is evaluated by pathologists, and the Gleason grading system is the cornerstone histological parameter for grading prostate cancers due to its' strong prognostic value. The ISUP grade group system is the currently applied refinement of the Gleason grading method [32]. The Gleason grade reflects the degree of differentiation of a sectioned prostate tissue sample and is progressively higher with loss of differentiation. Whereas Gleason grade 1 represents the most differentiated pattern which most closely resembles normal prostate architecture, Gleason grade 5 reflects the least differentiated pattern. The Gleason score (GS) yields the sum of the most predominant and second most predominant Gleason grades. Additional histological parameters with apparent prognostic value include presence and degree of perineural invasion, neuroendocrine differentiation [51] as well as intraductal carcinoma and cribriform architecture [31].

Clinical staging of prostate cancer is based on the TNM staging system [52] which assesses the extent of regional spread (cT1-4), lymph node involvement (N0/N1), and metastases (M0/M1). TNM stage,

PSA and ISUP grade grouping represents the current diagnostic and prognostic parameters used for treatment decision-making. These tumor-related characteristics can stratify patients into risk groups based on their retrospectively identified associations with recurrence following definitive treatment [10]. In patients deemed eligible for definitive radical prostatectomy (RP) with curative intent, tissue availability following surgical removal of the prostate and eventually seminal vesicles and/or regional lymph nodes allows for a more precise diagnosis and prognostic evaluation. Postoperative nomograms predicting disease recurrence following definitive therapy such as the CAPRA-S score [53] may include preoperative PSA, ISUP grade grouping, surgical margins, EPE, SVI, and lymph node involvement [54].

In Norway, 20-38% of prostate cancers diagnosed in 2017 were locally advanced [55]. At this stage, the disease is still considered curable, and is usually managed by radiotherapy in conjunction with androgen-deprivation therapy or prostatectomy. Upon evidence of metastatic spread to the lymph nodes or distant sites, palliative treatments are given to postpone disease-progression and relieve symptoms.

3.4. Prostate cancer treatment

Treatment options largely rely on the aforementioned risk group stratification. Besides, the benefit of clinical intervention is weighed against comorbidities associated with the treatment and the patient's life expectancy. To this end, watchful waiting represents a palliative, non-intervening strategy suitable for frail patients, where symptomatic treatment is deferred until deemed necessary. Active surveillance represents an option for active monitoring of a patient's disease where low or very low-risk prostate cancer is suspected, and possibly for intermediate-risk patients [10, 56]. Active surveillance thus aims to reduce overtreatment of tumors that are deemed to remain asymptomatic [10]. These latter patients are continuously candidates for curative treatment upon progression.

Personalized treatment is essential in the management of prostate cancer, as the choice of primary and secondary treatments has an impact on the sequential path of progression. Different intervention strategies throughout prostate cancer progression are introduced in this section.

3.4.1. Localized prostate cancer

Definitive RP has shown benefit over watchful waiting in terms of prostate cancer-specific survival (PCSM), particularly in young men (<65 years) and in intermediate-risk cancers (SPCG-4; [57]). Less than 20% of all Norwegian men diagnosed with prostate cancer were treated with definitive RP in 2004 [9]. Ten years later, nearly 40% of all diagnosed patients were radically operated. The increase in the use of RP has occurred due to more widespread use of the PSA test (leading to more low-PSA

(<10 ng/mL) diagnoses) and more use of RP in patients with high-risk disease. Definitive external-beam radiation therapy (EBRT) is an alternative curative treatment modality for localized prostate cancers. Although the European Association of Urology (EAU) does not recommend any active treatment modality over the other [10], a recent study reported EBRT to be inferior to RP in terms of overall survival [58]. Several alternatives to RP and EBRT exist, but data on long-term outcome for these procedures are lacking [10]. Neoadjuvant therapy such as androgen deprivation therapy (ADT) can in some cases be offered to reduce the primary tumor burden and potentially eliminate asymptomatic or non-visible metastases, thereby increasing the likelihood of successful localized treatment [59, 60]. However, several clinical trials conducted between 1996 and 2009 showed that although neoadjuvant ADT led to lowered positive surgical margin rates, lower tumor volumes and higher rates of organ-confined disease after RP, these pathological downgradings did not translate into improved PCSF or OS [60].

In a multicentre randomized clinical trial (RCT) initiated during the early years of PSA-testing, RP for low and intermediate-risk prostate cancer was associated with 91.5% cancer-specific survival at 19.5 years follow-up (PIVOT) [61]. In SPCG-4, conducted before widespread PSA-testing, nearly 20% of the radically operated patients had died from prostate cancer [62]. Aside from illustrating the generally favorable prognosis associated with low- and intermediate-risk prostate cancer, these RCTs also suggest that early prostate cancer diagnoses are associated with lowered PCSM.

3.4.2. Recurring disease following curative-intent treatment

Following definitive RP, 23-34% of the patients develop recurrent disease [10, 63, 64]. PSA levels are therefore closely monitored to detect persisting prostate cancer cells or recurrent cancer growth. Treatment failure is commonly defined as either (i) detectable PSA-levels directly after RP (≥ 0.1 ng/mL), or biochemical recurrence (BCR) determined as two sequential rises in PSA ≥ 0.2 ng/mL above nadir with at least one week between each measurement [10]. Patients with measurable PSA levels or rapid relapse following definitive treatment may be offered adjuvant therapy. Adjuvant therapy normally refers to either radiation therapy (RT) or ADT given within 6 months following curative-intent treatment. When biochemical recurrence is delayed, salvage treatment options are similar, and generally aim to eliminate or maintain the disease. Certain antiandrogens such as flutamide (Eulexin) and bicalutamide (Casodex) are offered in Scandinavian countries to retain biochemical control, normally to salvage treatment failure on RT. However, only weak evidence for a benefit of bicalutamide in the adjuvant or salvage setting following definitive treatment exists [10].

Despite efforts to control prostate cancer recurring following definitive treatment, around 10-13% of men experience failure on salvage treatment(s) and progress into advanced and eventually

metastatic disease [64]. Around 6% of patients progressing on salvage treatment(s) succumb to the disease [10]. In Norway, 5% of radically operated men between 2004 and 2011 developed metastatic disease following radical prostatectomy (personal communication, H. H. Grytli). While these numbers reflect the generally favorable prognosis of this patient group, they also underscore the importance of identifying patients with aggressive primary disease to offer treatment that can prevent or delay metastatic progression.

3.4.3. Locally advanced and metastatic hormone-naïve prostate cancer

Current guidelines state that men with locally advanced hormone-naïve prostate cancer (HNPC, sometimes termed "castration-sensitive") should be treated with concurrent local and systemic therapy [65]. It is currently not known which local treatment option is most efficacious, but both surgery and radiotherapy are commonly used, sometimes in combination with ADT, for non-frail patients. Metastatic HNPC (mHNPC) can either be determined as the primary diagnosis, which is most common, or may arise as a result of a patient failing non-hormonal therapy(ies) as described above. Androgen deprivation therapy is the preferred first-line treatment option for these patient groups.

Androgen deprivation therapy

ADT aims to lower the patient's systemic level of circulating testosterone and may be accomplished through either surgical or chemical castration. Chemical castration is the preferred method for achieving castration levels of testosterone today, and is accomplished by administration of LHRH agonists or antagonists. Both LHRH agonists and antagonists ultimately abrogate Leydig cell testosterone production and secretion. LHRH agonists, but not LHRH antagonists, lead to an initial flare in testosterone levels which may be handled with concomitant short-term AR antagonist (anti-androgen) administration (e.g. bicalutamide) [65], but both drug classes ultimately lower androgen levels. In this thesis, ADT exclusively denotes LHRH agonists or antagonists. Other drugs aiming to either antagonize AR or inhibit androgen biosynthesis are generally referred to as AR pathway inhibitors (ARPIs) of first- or second-generation. Overall, treatments aiming to limit AR signaling are denoted androgen-targeted therapies.

ADT has traditionally been used as a monotherapy for metastatic and rapidly progressing non-metastatic prostate cancer [65]. Although combination with first-generation AR antagonists (termed total androgen blockade) have been reported to offer a survival benefit, these treatments have been associated with more side-effects than ADT alone [66]. ADT may be given intermittently with the rationale that temporary cessation of ADT allows the cancer cells to better retain or regain AR dependency and therapeutic efficacy. Furthermore, there is a trend towards improved quality of life

in patients receiving intermittent ADT compared to continuous ADT [67]. Intermittent ADT is not routinely recommended, however, as large clinical trial [68] and meta-analyses ([65] and references therein) could not rule out inferior survival as compared to continuous ADT.

The treatment landscape for advanced prostate cancer has rapidly changed in recent years. While ADT remains the cornerstone, multiple drugs originally approved for CRPC have been or are being evaluated in RCTs enrolling patients with hormone-naïve (predominantly metastatic) lesions [69-73]. Within a treatment arm in the STAMPEDE trial, abiraterone (a steroid synthesis inhibitor; detailed in chapter 3.4.4) + ADT was associated with improved OS and progression-free survival compared to ADT alone [71]. These findings were supported by results from the LATITUDE trial [70]. The TITAN trial reported an OS benefit of adding the second-generation anti-androgen apalutamide to ADT in mHNPC [72]. Recent results from the ARCHES trial (NCT02677896) illustrated improved radiographic progression-free survival in men with hormone-naïve prostate cancer treated with ADT + enzalutamide (a second-generation anti-androgen; detailed in chapter 3.4.4) over ADT + placebo [73].

Many of these clinical trials are recent and the results are sometimes immature. This applies to e.g. the ARCHES trial where OS data are currently too scarce to be analyzed. It has not yet been assessed whether combination therapies (i.e. ADT+ARPI) are superior to sequential treatments in terms of OS. Specifically, the use of combinations may limit other choices once resistance manifests.

Around 70-80% of HNPC patients display symptomatic relief upon ADT [16], presumably due to the high dependency of prostate cancer cells to androgens [74]. Despite its' high efficacy, ADT alone or in conjunction with chemotherapy or ARPIs is associated with a predictable manifestation of castration-resistant prostate cancer (CRPC). A large variance in progression-free survival (PFS) on these treatments has been reported [42].

3.4.4. Treatment options for CRPC

Attributed to the commonality of continued AR signaling in most non-metastatic and metastatic CRPCs (mCRPCs), the currently preferred therapy for these patients involves drugs targeting the androgen signaling axis (ARPIs). Abiraterone (Zytiga) functions by inhibiting Cytochrome P450, Family 17, Subfamily A, Polypeptide 1 (CYP17A1), which catalyzes the hydroxylation of pregnenolone and progesterone as well as the subsequent formation of DHEA (Figure 3.4). Both DHEA and androstenedione are precursory metabolites to testosterone and DHT, and abiraterone thus inhibits intracrine androgen biosynthesis. Abiraterone was approved by the US Food and Drug Administration (FDA) in 2012 for use in men with mCRPC after the COU-AA-301 randomized, double-blind phase III study showed improved overall survival of abiraterone over placebo [75]. Enzalutamide (Xtandi) was

FDA-approved in 2014, and is a potent nonsteroidal anti-androgen that acts as a competitive inhibitor for the AR ligand-binding domain [44]. In the randomized, double-blind phase III PREVAIL trial [76], enzalutamide improved radiographic PFS and OS compared to placebo. Although several other antiandrogens such as bicalutamide have been used in the clinical management of prostate cancers, they are considered inferior to the mentioned ARPIs in the CRPC setting.

The novel antiandrogens apalutamide and darolutamide were both recently reported to offer around two-year increases in metastasis-free survival compared to placebo in double-blind, placebo-controlled phase III-studies for non-metastatic CRPC (SPARTAN [77] and ARAMIS [78], respectively). Both drugs were recently FDA-approved for use in this setting. Aside from ARPIs, other FDA-approved drugs shown to have clinical benefits in CRPC exist as well [79-82].

It is estimated that survival times after CRPC emergence is between two and three years [42], but these numbers are perceived to increase with the continued introduction of novel drugs, new indications for existing drugs and combination treatments.

3.5. Mechanisms of therapy resistance

Castration-resistant prostate cancer refers to a disease state in which tumor cells have escaped systemic androgen dependence. CRPC is defined as either three consecutive rises in PSA, with two rises at least 50% over PSA-nadir (PSAn; i.e. the lowest achieved PSA), and a PSA > 2.0 ng/mL with at least one week between each measurement, or radiologic progression, despite castrate serum levels of testosterone (<1.7nmol/L [50 ng/dL]) [65].

There are currently two main hypotheses proposed to explain how CRPC develops, and the two are not known to be mutually exclusive: Whereas the clonal selection hypothesis refers to cancer cells with stem/basal-like properties being selected for during ADT due to their inherent androgen indifference, the adaptation hypothesis refers to cancer cells adapting to a low-androgen tumor microenvironment. ADT is increasingly used in combination with ARPIs at different disease stages, and to what extent and eventually how cross-resistance manifests is not well described to date. Most mechanisms of resistance to ADT have been described in the era of ADT monotherapy. As mentioned, ARPIs (see chapter 3.4.4) have traditionally been administered to patients with CRPC, but are now increasingly used in combination with ADT for HNPCs. Several resistance mechanisms to ADT and ARPIs are perceived to overlap, as reactivation of the AR signaling axis commonly occurs under resistance from both intervention strategies.

3.5.1. Mechanisms involving the androgen receptor pathway

CRPC is not a hormone-refractory disease like its' preceding term hormone-refractory prostate cancer implies: For most patients, CRPC represents a disease state in which the androgen signaling axis remains active [44], emphasized by the broad clinical use of PSA as an early biomarker for CRPC emergence.

AR signaling remains important in most advanced prostate cancers, including the majority of, but not all, CRPCs [44, 83]. As opposed to the healthy prostate where AR stimulation promotes terminal, luminal differentiation [84, 85], AR signaling can elicit both tumor-suppressive and oncogenic effects in prostatic adenocarcinoma cells. This indicates that the effect of AR is contextual. When the supply of androgen is limited, prostate cancer cells may maintain AR signaling through multiple mechanisms that can confer androgen hypersensitivity. In brief, these mechanisms are perceived to occur upon treatment pressure, which promotes gain-of-function mutations, *AR* gene amplification, AR cross-talk with multiple pathways, chromatin remodeling, and alterations in the steroid biosynthetic pathway. These mechanisms are further detailed below.

The continued AR signaling observed in most ADT-treated prostate cancers implies an intrinsic dependence on AR signaling and its associated pathways which should be considered when studying resistance mechanisms towards AR-targeted therapies.

3.5.2. Intracrine androgen biosynthesis

Dillard *et al.* reported that prostate cancer cell lines are able to perform intracrine androgen biosynthesis from the early precursory metabolite cholesterol [86]. Cholesterol can be produced from acetyl coenzyme A in prostate cells [87] or taken up from the blood [46]. Compellingly, androgen levels have been shown to increase during CRPC development after castration in an androgen-dependent LNCaP xenograft model [87]. The intratumoral testosterone concentration in castrated prostate cancer patients was higher than in noncancerous control tissues and primary, non-castrated prostate cancer tumors [88], thus emphasizing also a clinical relevance. Together, these reports indicate that prostate tumors are capable of complete intracrine synthesis also in absence of bioavailable testosterone and its precursory, adrenal cortex-derived steroid metabolites.

Prostate cancer cells are known to express steroid 5α reductase enzymes (SRD5A1 and SRD5A2) ([89] and references therein). These enzymes catalyze the conversion of testosterone into the more potent DHT [44] (Figure 3.4). Also, both CRPC tissue and cell lines may synthesize DHT from 5α-androstanedione via SRD5A1-mediated 5α-reduction of androstenedione [90], thereby bypassing testosterone and alleviating testosterone dependency under androgen-depleted conditions. The complexity of the steroidogenic pathway illustrates the availability of multiple pathways for the

production of testosterone and DHT, but also underlines the need for broad targeting to achieve biochemical inhibition of steroidogenesis.

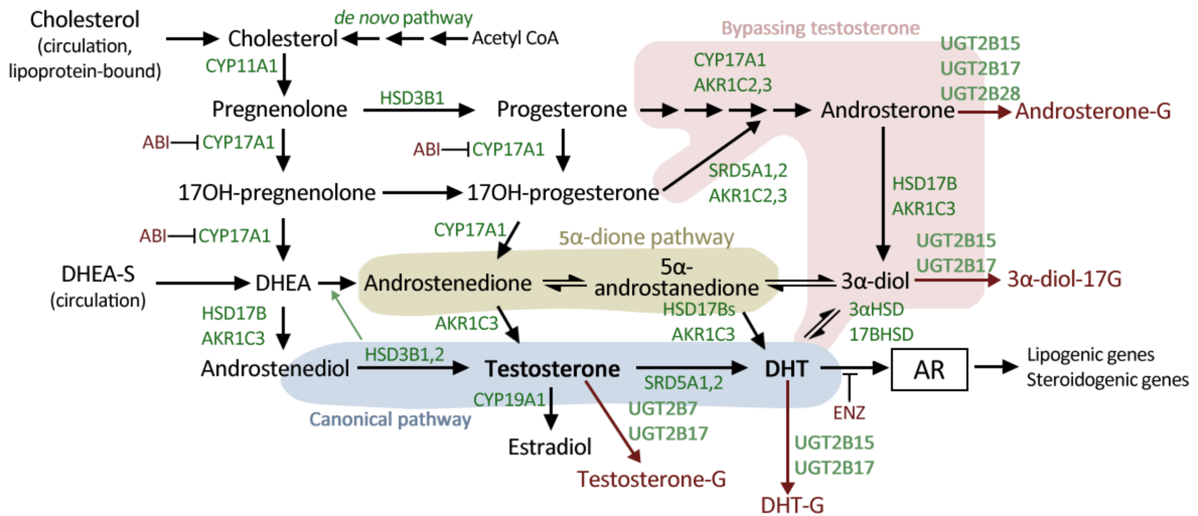


*Figure 3.4. **The steroidogenic pathway.** Based in part from [91] and [92]. Endogenous or de novo synthesized cholesterol is converted to pregnenolone by CYP11A1, and further to DHEA in a two-step manner catalyzed by CYP17A1 (target enzyme of abiraterone acetate (ABI)), yielding DHEA. DHEA may also be taken up as DHEA-sulphate (DHEA-S) of adrenal origin. DHEA may, in turn, be converted to either androstenediol (canonical pathway) or androstenedione (5α-dione pathway), two testosterone precursors. Pregnenolone and its' 17-hydroxy metabolite can be converted to progesterone, which further may be converted to androsterone in a series of biochemical steps. Androsterone may be converted to 3α-androstanediol (3α-diol), which can yield DHT directly, thereby bypassing testosterone ("backdoor pathway"). DHT can activate the AR, but this interaction may be challenged by enzalutamide (ENZ) administration. The AR regulates lipogenic genes involved in e.g. cholesterol biosynthesis, as well as steroidogenic enzymes. Both androsterone, 5α-androstenedione, 3α-diol, testosterone and DHT may be irreversibly converted to their glucuronide conjugates (e.g. DHT-G) by the UGT2B-family of enzymes (references in text).*

In the mentioned LNCaP xenograft CRPC model [87], mRNA levels of several key steroidogenic enzymes were upregulated during CRPC progression. Metastatic CRPC tumors transcriptionally overexpress many of the same steroidogenic enzymes (such as CYP17A1, a target of the antiandrogen abiraterone; Figure 3.4), and display a higher DHT/testosterone ratio compared to primary (non-castrated) tumors [88]. The clinical efficacy of drugs targeting the steroidogenic pathway illustrates the dependency and vulnerability of prostate cancer cells to intratumoral androgen metabolism.

Abiraterone prevents intracrine production of adrenal androgens from cholesterol, and should therefore theoretically inhibit androgen biosynthesis via the "5α-dione" pathway (in which testosterone is bypassed) [90], aside from its canonical function towards CYP17A1. During abiraterone treatment, cells may escape treatment through various mechanisms: Overexpression or mutations in *CYP17A1* have been shown to alleviate the antagonistic effects of abiraterone ([44] and references therein), and around 20% of tumors that had progressed upon combinatory abiraterone and glucocorticoid treatment harbored *AR* mutations which enable glucocorticoids to act as AR agonists [93].

3.5.3. Intratumoral androgen elimination

Intraprostatic androgen concentrations in CRPC tumors were reported to be sufficiently high to sustain AR signaling [88]. This may not exclusively relate to intracrine androgen biosynthesis, but also decreased elimination of androgens. Prostate cancer tumors and cell lines express enzymes that participate in the elimination of bioavailable androgens [94]. The UDP-glucuronosyltransferase 2B (UGT2B) family of proteins comprise a group of enzymes that participate in the phase-II metabolic glucuronidation of metabolites through the transfer of a glucuronic acid (UDPGA) moiety. The UGT2B proteins show specificity for androgens as well as other hydrophobic molecules [95-97]. Glucuronidated androgens are functionally inactive [96]. UGT2B15 and UGT2B17 show particularly high expression in prostate cancer tumors and cell lines, and both participate in the irreversible glucuronidation of testosterone and DHT, as well as other steroid metabolites including 3α-diol and 5α-androstenedione [97, 98] (Figure 3.4).

Both the *UGT2B15* and *UGT2B17* genes contain AREs in their promoter regions, and are predominantly expressed in AR-positive cell lines [99]. Accordingly, studies on LNCaP cells have revealed that DHT-mediated AR stimulation reduces UGT2B15 and UGT2B17 mRNA levels [99], whereas androgen depletion increased at least the UGT2B17 protein levels in another study [100]. Although overexpression of an androgen-eliminating enzyme, particularly in the ADT setting, would imply lowered androgen bioavailability and AR signaling, Li *et al.* illustrated that UGT2B17 stimulated proliferation and invasion *in vitro* and promoted CRPC development in a xenograft model [100]. Using clinical samples, one study found that having double deletions in the *UGT2B17* gene was associated with shorter BCR-free survival, whereas UGT2B15 and UGT2B17 mRNA levels were more recently reported to show no such associations [101].

Paquet *et al.* showed that UGT2B15 protein levels were reduced in primary and CRPC tumors [102]. Conversely, two other studies reported elevated UGT2B15 protein levels in ADT-treated tumors and transcriptional upregulation in CRPC tumors compared to primary tumors and benign samples [88,

94]. Protein levels of UGT2B17 have been reported to be upregulated in primary and CRPC tumors compared to benign samples, and further upregulated in lymph node metastases [102]. Like UGT2B15, UGT2B17 transcript levels are reportedly higher in mCRPC samples compared to primary tumors and benign samples [88], whereas UGT2B17 protein levels were only temporarily increased during ADT and returned to levels similar to that of primary tumors and benign samples upon CRPC development [94]. The latter findings conflict a larger study (comprised of samples studied in [102] and [94]) which showed elevated UGT2B17 protein levels in both CRPCs and primary tumors treated with neoadjuvant ADT [100]. In the latter study, UGT2B17 was also found to be associated with GS.

A factual prognostic and predictive value of differential UGT2B expression thus remains unclear. A biphasic relationship between AR signaling and aggressive prostate cancer was recently suggested [103]. Furthermore, supraphysiological testosterone treatment has been shown to invoke clinical responses in men with HNPC and CRPC [84]. This implicates that contextual considerations towards different disease stages and prior treatments should be taken when interpreting the clinical significance of these enzymes. Finally, UGT2B7 has been reported to show enzymatic activity towards bicalutamide [96], and the high homology of the *UGT2B* genes [104] indicates that elimination of targeted therapeutics may also affect clinical outcomes in advanced prostate cancer.

3.5.4. *Regulation of nuclear AR activity*

Mutation rates are generally low even in advanced prostate cancer [105], while metastatic CRPCs display a higher mutational burden than hormone-naïve metastases [34]. This suggests a progressively increasing mutational burden during disease progression. *AR* amplifications and mutations are uncommon in untreated tumors but are highly frequent in treatment-challenged mCRPCs [106-108]. AR activation leads to upregulated expression of DNA damage repair genes, a finding that is also apparent in mCRPCs ([109] and references therein). These studies may reflect the general increase in the mutational burden in therapy-resistant tumors with aberrant AR activity. Unsurprisingly, mechanisms of resistance towards enzalutamide exist: *AR* point mutations have been shown to lead to a switch in which enzalutamide becomes an AR agonist in enzalutamide-resistant LNCaP cells [110, 111]. Furthermore, specific *AR* mutations associate with resistance to bicalutamide [112, 113]. Several of these mutations are perceived to explain antiandrogen withdrawal syndrome in which patients discontinuing e.g. bicalutamide display an initial decline in PSA levels [114]. This characteristic implies that *AR* mutations can facilitate antiandrogens to function as AR agonists, as has been demonstrated in LNCaP cells undergoing long-term androgen-depletion [115].

AR amplifications have been shown to positively correlate with AR gene expression [107], and are assumed to associate with elevated sensitivity towards androgens [116, 117]. *AR* mutations

commonly arise in the ligand-binding domain (LBD), which may permit binding of ligands normally lacking affinity for the AR such as DHEA, glucocorticoids, and progesterones [42, 93]. Aside from AR gene amplifications, mutations and interacting signaling molecules, several AR splice variants (AR-Vs) are well documented, such as AR-V7 [42, 44]. In fact, at least 22 AR-Vs have been documented in CRPC tumors [106]. The alternative splicing commonly results in translation of AR proteins lacking the LBD which makes them constitutively active. AR-V7, as well as AR-V1, were found to be significantly elevated in CRPC compared to primary, ADT-naïve cancer tissue [118]. A more recent study reported that AR-V3, AR-V7, and AR-V9 are more frequent in CRPC tissue as compared to primary hormone-naïve tumors, and nearly 70% of the CRPCs harbored all three variants [119].

Like in the setting of ADT, AR-Vs are perceived to confer resistance to ARPIs [120, 121], although controversies exist [122, 123]. The presence of the most well-studied AR-V7 variant in non-invasive liquid biopsies has been associated with poor clinical outcomes in patients treated with abiraterone and enzalutamide [120, 124, 125]. Multiple AR-Vs have been demonstrated to limit enzalutamide's effect towards inhibiting androgen-driven nuclear translocation of full-length AR [126]. Furthermore, LNCaP cells with AR-V7 overexpression were more bicalutamide-resistant than control cells [127]. In the same study, AR-V4 and AR-V6 promoted castration resistance through driving the expression of canonical full-length AR target genes. AR-Vs were recently shown to bind to AREs on enhancer elements within open regions of chromatin in CRPC cells predominantly without requiring pioneering Forkhead Box A1 (FOXA1) binding and drove expression of genes up-regulated in abiraterone-resistant CRPC tumor specimens [128].

A wide range of molecules are known to act as co-activators or co-repressors of the AR, and the molecular functions of these AR coregulators vary [40, 44]. AR coregulators allow for intricate control of transcription of specific genes or sets of genes upon AR activation. Briefly, coactivators function by permitting the formation of an AR-containing transcriptional complex through e.g. chromatin remodeling, ultimately engaging RNA polymerase II to ARE-containing gene promoters [40, 129]. Corepressors can suppress AR activity through e.g. preventing coactivator binding or direct AR binding and recruitment of chromatin remodeling enzymes that prevent target gene transcription [130]. An open chromatin structure is necessary for a successful establishment of transcriptional complexes and subsequent gene transcription [131], and multiple AR coregulators, and the AR itself, act on the chromatin structure. Differential regulation of AR coregulators during disease progression enables highly contextual and diverse expression patterns [132], and dysregulated expression of AR coregulators has been associated with poor outcomes in prostate cancer. Multiple AR coregulators are concomitantly transcriptionally regulated by the AR [132]. AR coregulators are typically involved in multiple pathways, such as DNA damage repair, kinases, chromatin remodeling, histone

modification and many more (reviewed in [129]). Importantly, many of the AR coactivators are upregulated in advanced prostate cancer, including CRPC, as compared to low-grade disease [44], while co-repressor proteins typically are downregulated.

Aberrant levels of AR coregulator proteins and TFs lead to an altered AR cistrome (i.e. the complete set of AR binding sites), which can drive disease progression in a feed-forward manner by e.g. altering transcription of multiple TFs. In particular, MYC Proto-Oncogene, BHLH Transcription Factor (c-MYC), glucocorticoid receptor (GR) and the mentioned AR-Vs have been recognized as important drivers of the emergence of conventional CRPC adenocarcinoma. Arora and colleagues reported that the GR can bypass AR antagonism through putative binding to AR target genes that share binding sites for AR and GR [133]. In cell line models, c-MYC was shown to drive AR expression and promote stability of different AR isoforms, and c-Myc levels were correlated with expression of both full-length AR and AR-Vs in CRPC specimens [134].

The mentioned AR coactivators and corepressors make out only a small portion of the intricate AR regulatory network that is beyond the scope of this thesis. Thorough reviews have been published on the matter [40, 42, 44].

3.5.5. AR cross-talk with signal transduction pathways

Multiple signaling pathways that cross-talk with the AR are aberrant in CRPCs [42]. These pathways can stimulate AR activity in a ligand-independent manner and sensitize the receptor for ligand activation [135, 136]. Hence, these pathways represent putative mechanisms facilitating resistance to ADT [42, 137]. Insulin-like growth factor-I (IGF-I), keratinocyte growth factor (KGF) and epidermal growth factor (EGF) can, like androgens, drive AR target gene transcription [138]. The phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway has also been implicated to cross-talk with the AR: Loss of Phosphatase and tensin homolog (*PTEN*), which is frequent in prostate cancers [139], leads to activation of the PI3K/AKT pathway, and can suppress AR transcriptional activity and promote growth independently of AR signaling [140]. The cytokine interleukin-6 (IL-6) can synergistically activate AR under low androgen concentrations [141]. IL-6 can also activate AR in a ligand-independent manner through the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, but the effect is presumably contextual and dependent on AR status ([136] and references therein).

The β-catenin protein partakes in the Wnt signaling cascade. Co-localization of AR and β-catenin proteins has been shown to be elevated in CRPCs [142], and β-catenin synergizes with androgens to increase AR activity [143]. As reviewed by Schneider and Logan, other members of the Wnt pathway can confer AR transactivation [144]: For example, glycogen synthase kinase β (GSK3β), which when

active tags β-catenin for proteosomal degradation by phosphorylating its amino-terminal region [145], can repress AR transactivation through phosphorylation of the AR amino-terminal region [146, 147].

Agents promoting increased intracellular cyclic AMP (cAMP) levels, such as β-adrenergic receptor agonists (β-agonists), can activate AR-DNA binding in AR-expressing cell lines and prostate explants both synergistically and in absence of ligand via cAMP-dependent protein kinase A (PKA) [148-151]. Accordingly, androgen-independent prostate cancer tissue specimens and cell line models have been shown to express low levels of phosphodiesterase 4D7 (PDE4D7) which cleaves cAMP [152]. Activated PKA can phosphorylate the AR at Ser-650 [153], although it is debated whether the AR in fact contains PKA recognition sites [154]. Furthermore, active PKA signaling was more recently shown to be essential for heat shock protein 90 (HSP90) phosphorylation [155], which alongside the putative PKA-mediated AR phosphorylation facilitate AR nuclear translocation [137]. Finally, activation of the β-adrenergic signaling axis is abundantly documented to activate the AR [151, 156, 157].

3.5.6. Androgen-independent/AR-indifferent ADT resistance mechanisms

Despite the crucial role of AR in prostate cancer development, progression and therapy resistance, a subset of tumors completely bypass the AR and androgen signaling by relying on alternative growth and survival pathways [158], exemplified by attenuated AR signaling in these cells [159].

The mechanisms conferring such AR-independent/indifferent resistance are not well understood, but are perceived to occur via cellular plasticity. Cellular plasticity may involve reversible or irreversible lineage switching, which allows a cell to alter its 'identity' to tackle treatment challenge and to promote metastasis. In the context of prostate cancer cells developing AR-indifference, the cells have seemingly fully lost their prostatic luminal identity and often acquired stem-like, epithelial-mesenchymal transition (EMT), neuronal and/or NE features through what is commonly termed lineage plasticity [159]. To what extent lineage plasticity occurs upon treatment pressure with ADT monotherapy, and whether it is an inherent capability of a subset of prostate cancer cells, is not well known. What is known, however, is that the prevalence of tumors displaying lineage plasticity has increased and coincided with more widespread use of AR pathway inhibitors in advanced prostate cancer care [20, 33, 83, 160, 161].

3.6. Lineage plasticity and therapy resistance

AR pathway inhibitors have offered a survival benefit for men with CRPC. With novel AR-targeted drugs, as well as new indications for their use, it is believed that men with lethal prostate cancer will experience prolonged survival. As introduced in the preceding chapter, the molecular mechanisms conferring resistance to androgen-targeted therapies largely invoke re-activation of the androgen signaling axis. In these cases, like before treatment, the recurrent tumors are histologically determined as adenocarcinomas (CRPC adenocarcinomas). More widespread use of ARPIs such as abiraterone and enzalutamide have however increased the frequency of CRPCs that do not rely on the AR to survive and progress [24, 83, 160, 162]. These prostate cancer subtypes frequently show histological characteristics unreminiscent of their native adenocarcinoma lineage, suggesting that they have rewired their identities through lineage plasticity.

3.6.1. Prevalence of androgen-independent CRPC subtypes

Castration-resistant prostate cancers not reliant on AR signaling can be termed androgen-indifferent CRPCs (AI-CRPCs) [163]. The majority of these subtypes occur with resistance towards combination treatments such as ADT+ARPI. These tumors are commonly AR-negative and express disproportionately low PSA, and their AR-indifference suggests that they rely on alternative pathways for sustained survival during ADT and/or ARPIs [24], as described later. AI-CRPCs are believed to arise through selective pressure on cancer cells which can lead to adaptive and/or selection mechanisms that alleviate androgen dependence [24, 33, 83]. Although a proportion of these CRPC variants are ARPI- and chemotherapy-naïve (i.e. diagnosed in recurrent CRPCs without second-line treatment), they are more frequent in men who have undergone second-line AR pathway inhibition [164]. This suggests that heavy and multimodal targeting of AR signaling can enforce androgen independence. The frequency of AR-negative CRPC variants with or without neuroendocrine differentiation (NED) is expected to further increase with more widespread use of ARPIs and the introduction and implementation of novel, more potent ARPIs [33, 159] (Figure 3.5).

In Norway, AI-CRPCs with NED are relatively rarely diagnosed and the majority of metastatic CRPC biopsies are histologically determined adenocarcinomas (personal communication with Dr. K.M. Russnes (Akershus University Hospital)). Post hoc analyses of the PREVAIL study performed in the US showed that up to 25% of patients diagnosed with metastatic prostate cancer undergoing ADT experienced radiographic progression without a rise in serum PSA [165]. In keeping with the findings from large clinicogenomic studies performed in the US [83, 164], these AI subtypes may be underdiagnosed in Norway. Tumors showing castration-resistant progression without biochemical recurrence are not routinely biopsied in Norway. In cases where the PSA is disproportionately low

and the patient has an unusual metastatic spread, however, biopsies are occasionally taken. But even here, only a minority show NED (Dr. K.M. Russnes). It is plausible that only large-scale studies on metastatic biopsies or autopsies from patients treated with multimodal AR-targeted therapies will reveal a more factual prevalence of this disease subtype in Norway.

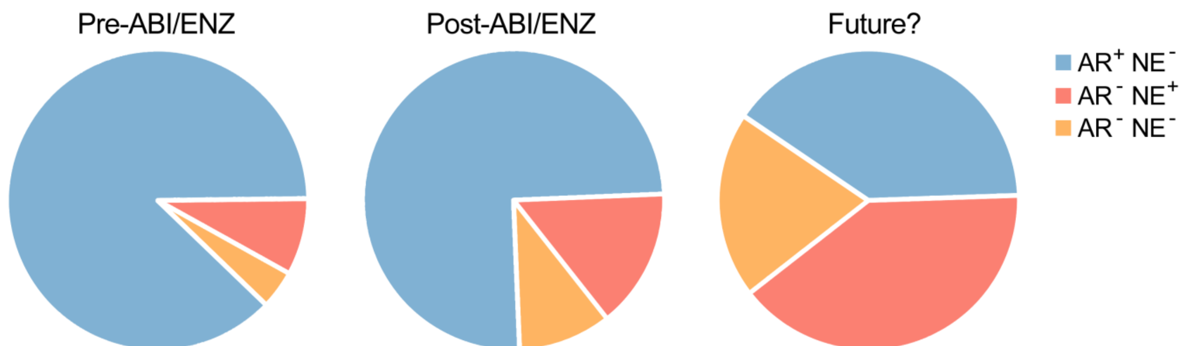


Figure 3.5. ***An approximate distribution of CRPC subtypes*** *(AR^+NE^- and AI-CRPCs with or without NED) in the pre-abiraterone (pre-ABI)/enzalutamide (ENZ) and post-abiraterone/enzalutamide era (~2012-) in the United States [83, 161, 164]. A suggested distribution for the future with more widespread ARPI administration and the introduction of novel ARPIs that further extend survival times of men with CRPC is also shown. NE = neuroendocrine.*

3.6.2. Clinical and molecular characteristics of AI-CRPCs

The most well-studied subgroup of AI-CRPCs is the AR-negative tumors with NED and small-cell histology. These variants are commonly termed treatment-induced neuroendocrine prostate cancer (t-NEPC) and make out a significant proportion of AI-CRPCs, as introduced later. The terms NEPC, t-NEPC, and CRPC-NE are often used interchangeably and irrespective of hormone-naivety. For clarity, t-NEPC will here be used to denote CRPC tumors of adenocarcinoma clonal origin that develop histological and clinical evidence of NEPC following AR targeted therapy(ies). *De novo* or primary small-cell carcinoma of the prostate (SCCP) here denotes the rare cases of primary diagnosed prostate cancers with small cell/neuroendocrine histology.

Treatment-related NEPCs show extensive NED which is determined by positive immunohistochemical staining of neuroendocrine markers such as CHGA, synaptophysin (SYP) and/or neuron-specific enolase (NSE/ENO2) [166]. NED is not exclusively found in AI-CRPCs, however, but is commonly observed as scattered NE-positive cells or more widespread as focal NED in untreated prostate cancers with adenocarcinoma histology [2]. Thus, a clinical diagnosis of t-NEPC requires additional investigations: The phenotype and clinical course of t-NEPC are strikingly similar to that of *de novo* SCCP which makes out <2% of all prostate cancer diagnoses [167]. Like SCCPs, t-NEPCs typically

display visceral metastatic spread, osteolytic bone metastases and associate with particularly poor outcomes [160, 168]. Such SCCP-like features, along with treatment history, disproportionally low PSA, and NED can substantiate a t-NEPC diagnosis.

Sainio *et al.* reported that as many as 80% of mCRPC tumors express at least focal NED admixed in adenocarcinoma [169], which is far higher than the reported frequency of t-NEPCs. Focal NED was found to be highly heterogeneous in regard to the distribution of positive NE-markers. In CRPCs with focal NED, AR expression levels were similar to NE-negative CRPCs, while strong expression of NSE and CHGA associated with AR-negativity [169]. Whether focal NED precedes t-NEPC is not known. A recent meta-analysis showed that focal NED associated with BCR after curative-intent RP [170], but the prognostic value of NED remains controversial [169] and may be influenced by the lacking consensus in grading of NED.

Aside from t-NEPC, a recent study identified double-negative prostate cancers (i.e. negative for both AR and NE-markers) [83]. Additional subtypes have been proposed to exist as well [159], and multiple variants can exist within distinct metastases in oligometastatic disease [171] and coexist within a tumor. Treatment-related NEPC tissue is frequently admixed with tissue components with an architecture reminiscent of adenocarcinoma (termed amphicrine tumors) [159]. It is currently not known whether the various AI-CRPC subtypes represent a continuum of phenotypes or distinct entities, but t-NEPC has been suggested to represent the most aggressive subtype [172], and may thus represent the final stage of lineage plasticity.

Intriguingly, high grade, therapy-resistant tumors with neuroendocrine features are not restricted to the prostate [173]. Multiple human epithelial adenocarcinomas from different origins become increasingly independent of their origin, and rather converge to a common, lethal small-cell phenotype, during progression on targeted therapies [173]. A particular resemblance to t-NEPC is found in small-cell lung cancers [174] which may arise *de novo* or as a result of potent targeted therapy [175].

Among chemotherapy-naïve CRPC-AIs in the post-abiraterone/enzalutamide era in the United States, 13-27% of patients have one or more lesions displaying t-NEPC (i.e. AR^{-}/NE^{+}; Figure 3.5) [83, 161, 164, 176], a significant proportion display AR^{-}/NE^{-} phenotypes and the majority display AR^{+}/NE^{-} (i.e. conventional CRPC adenocarcinoma) [83]. Both clonal selection of clonally-derived lineage plastic cells and adaptive, treatment challenge-associated mechanisms have been proposed to explain the emergence of t-NEPCs and thus why a subgroup of tumors respond poorly to ADT and/or ARPIs [177].

3.6.3. Molecular evolution of t-NEPC

Lineage plasticity is increasingly used to describe how prostate cancer cells can alleviate androgen dependence and become therapy-resistant [20]. A recent workshop on lineage plasticity concluded that the underlying mechanisms driving lineage plasticity are unclear [159]. Aside from the intermediate stem-like transition, EMT and direct transdifferentiation (introduced later) were put forward as potential mechanisms. The latter two do not necessarily rely on stem-like reprogramming.

NE cells in the normal prostate have been suggested to be of neurogenic origin (i.e. derived from the neural crest) [178], which would indicate that they are of a different lineage than basal and luminal cells. In contrast, enrichment of a basal stem cell signature has been reported in t-NEPCs and *de novo* [179], and basal cell signatures are enriched in metastases and NEPCs [179, 180]. Lee and colleagues recently showed that inhibition of the tumor suppressor functions of RB Transcriptional Repressor 1 (RB1/pRb), Tumor Protein P53 (p53) and protein phosphatase 2 (PP2A) in $p63^+$ basal-like progenitor cells promoted NEPC development in mice [181]. Although this could imply that NEPCs are of basal cell (AR^-/PSA^-) origin, the transmembrane protease, serine 2:ETS-related gene (*TMPRSS2-ERG*) gene fusion (present in ~50% of all primary prostate cancers [182, 183]) has been shown to be concordant between admixed SCCPs and adenocarcinomas [184], thus suggesting an adenocarcinoma lineage.

While the majority of t-NEPCs are perceived to arise in CRPCs treated with ARPIs, both NE and NEPC features are found in abiraterone/enzalutamide-naïve mCRPCs [164, 169]. Hence, at least a subset of t-NEPCs may arise during ADT. Studies on autopsy-derived CRPC metastases and preclinical models have shown that t-NEPC arises through divergent clonal evolution [33, 185]. This would indicate that conventional adenocarcinoma CRPCs transdifferentiate and yield clones displaying t-NEPC (thus indicating a luminal-to-basal lineage switch rather than clonal selection). In this regard, transdifferentiation may enable tumor cells to circumvent drugs targeting the androgen signaling axis [24, 33, 185]. However, a mechanistic explanation involving clonal selection of hormone-naïve lineage plastic cells has not been rejected thus far, and it is not known if the two mechanisms may function in concert or as consecutive events (Figure 3.6).

Pathways driving t-NEPC emergence and maintenance

A genome-wide DNA methylation analysis of autopsy-derived CRPC tumors performed by the Beltran lab at Weill Cornell showed that t-NEPCs were epigenetically highly divergent from their CRPC adenocarcinoma counterparts [33]. In particular, they were characterized by epigenetic dysregulation of stem cell programs, neuronal, and EMT pathways, resulting in altered gene expression. In the same study, deletions in *RB1* and *TP53* were two-fold more common in t-NEPCs than CRPC adenocarcinomas individually, and concurrent deletions were enriched nearly four-fold

[33]. Other studies have reported *RB1* loss in 90% of NEPC/SCCP cases, as compared to 74% in metastases and CRPC adenocarcinomas and 34% in primary tumors [186, 187]. Conversely, four patient-derived organoid xenograft models with t-NEPC features were recently shown to display loss of RB1 pathway function without genetic *RB1* loss [188]. *TP53* and *RB1* dysregulation has also been reported in lung and breast cancers with small-cell histology [173]. Together, these studies are suggestive of a progressive accumulation of *RB1* and/or *TP53* deletions or loss of function resulting from targeted treatment challenges.

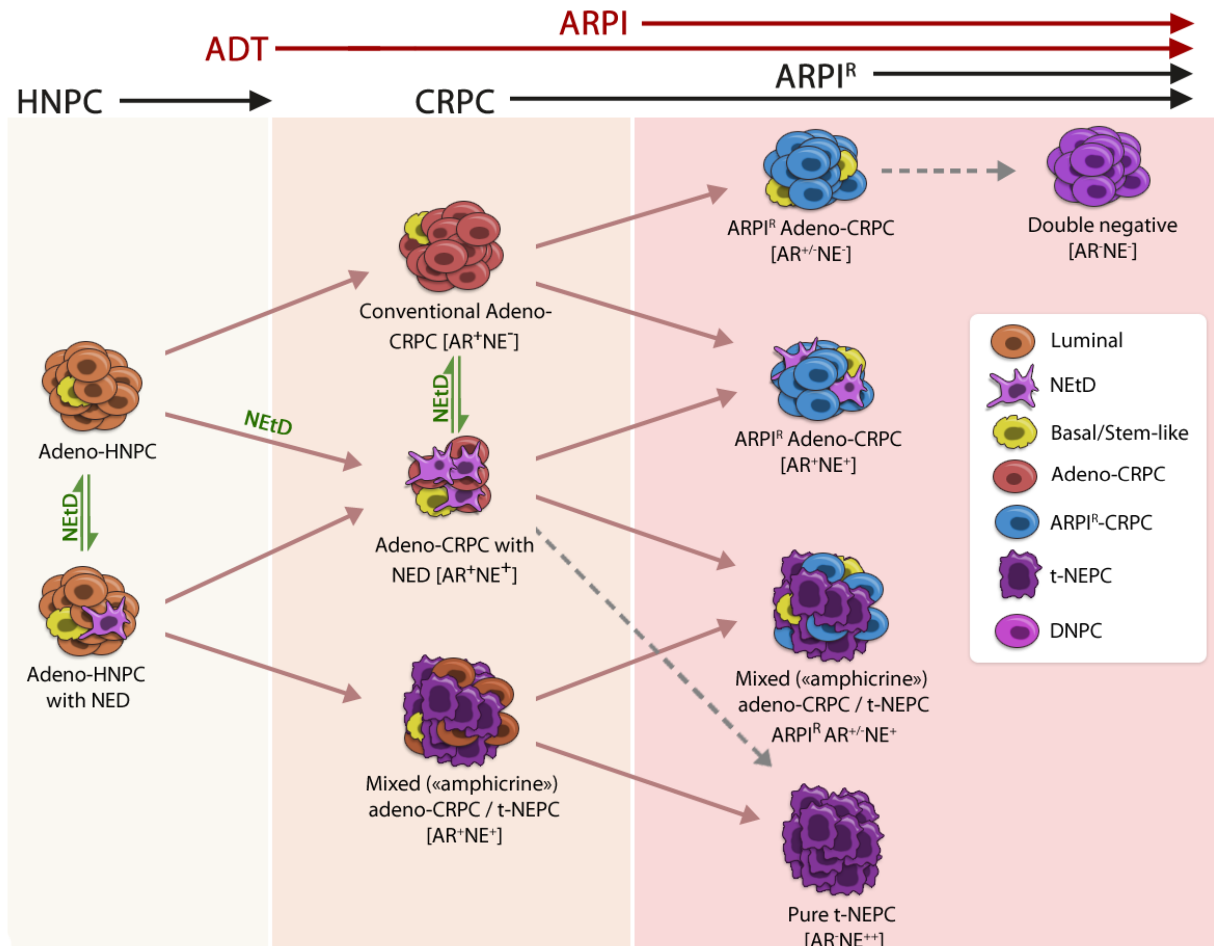


Figure 3.6. ***A hypothetical model for the evolution of prostate cancer subtypes resistant to ADT and ARPIs.*** *Hormone-naïve, well-differentiated prostate cancer adenocarcinomas (HNPC) can exist with or without neuroendocrine differentiation (NED) and likely also basal/stem-like cell populations. NE cells may derive from neuroendocrine transdifferentiated (NEtD) luminal cells or another cell lineage. Androgen deprivation therapy (ADT) may, aside from promoting CRPC adenocarcinoma, favor NEtD and possibly drive t-NEPC emergence alongside with CRPC adenocarcinoma ("amphicrine"/mixed phenotype). The addition of ARPI to ADT can further drive the emergence of ARPI-resistant CRPC adenocarcinoma with or without NED from NE-positive and NE-negative ARPI-naïve CRPC adenocarcinomas, and possibly also double negative prostate cancer (DNPC). ARPI-treatment of CRPC adenocarcinomas can yield focal NED, admixed adenocarcinoma/t-NEPC by clonal expansion*

of NE-transdifferentiated cells having acquired loss-of-function in critical tumor suppressor genes, and possibly pure t-NEPC.

In a preclinical study, *PTEN*-deficient mice, which spontaneously develop prostate adenocarcinoma in an RB1 (*PTEN*$^{-/-}$ *RB1*$^{-/-}$) or *TP53* background (*PTEN*$^{-/-}$ *RB1*$^{-/-}$ *TP53*$^{-/-}$), displayed elevated expression of NEPC/SCCP-related genes relative to single knockout mice (*PTEN*$^{-/-}$) [189]. Mu and colleagues reported that stable dual knockdown of TP53 and RB1 in LNCaP cells led to elevated expression of NE genes, lowered AR gene expression and enzalutamide resistance, indicating that *TP53* and *RB1* loss confers lineage plasticity [190].

Immunohistochemical staining revealed a marked and progressive elevation of the epigenetic modulator enhancer of Zeste homolog 2 (EZH2; the functional protein in the polycomb repressive complex 2 (PRC2) [191, 192]) and the lineage pluripotency SRY-box transcription factor 2 (SOX2) in double and triple knockout mice [189]. Supportive of a clinical relevance of these models, EZH2 is reportedly upregulated in t-NEPCs with a concomitant decrease in expression of EZH2 target genes [33]. Aside from functioning as a transcription factor (TF) driving AR transcription, EZH2 is downregulated by androgens via RB1 and p130 [193], and it coordinates transcriptional repression through catalyzing the deposition of trimethyl marks at H3K27s (H3K27me3) at target genes' promoter regions [194, 195]. Although it remains largely unknown how EZH2 contributes to the emergence of t-NEPC, H3K27me3-levels have been reported to be higher in NEPC cell lines (such as NE1.3 and NCI-H660) than in more well-differentiated/adenocarcinoma prostate cancer cell lines [196]. In the study by Ku and colleagues, EZH2 silencing and inhibition restored sensitivity to enzalutamide in the *PTEN*$^{-/-}$ *RB1*$^{-/-}$ *TP53*$^{-/-}$ mouse model [189]. Beltran *et al.* showed that the AR-negative, small cell/NEPC NCI-H660 cell line was sensitive to EZH2 inhibition [33], indicating a clinical utility of targeting EZH2 in t-NEPC.

Mu *et al.* showed that SOX-2 is a critical mediator of the increase in lineage plasticity upon TP53/RB1 knockdown [190]. Furthermore, SOX2 has been shown to drive expression of neural differentiation genes during hippocampal neurogenesis [197], and RB1 epigenetically repressed transcription of SOX2 and POU Class 5 Homeobox 1, POU5F1Oct4 (Oct4; another stem cell pluripotency TF) [198]. Taken together, these findings support a model in which loss of critical “gatekeeping” tumor suppressor genes (TSGs) leads to aberrant transcriptional regulation of genes modulating lineage plasticity through epigenetic dysregulation and transcriptional reprogramming [33, 189, 190, 198]. Although the recent study by Lee and colleagues suggested that NEPCs are of basal/progenitor origin [181], it could well be that the gatekeeping tumor suppressors function similarly in basal/progenitor-like and luminal-like cells.

The promising results from a clinical study using targeted inhibition of aurora kinase (AURKA) for patients with t-NEPC have underlined the importance of these pathways in t-NEPC [199]. AURKA promotes stabilization of the N-Myc protein [200], and gene amplifications of both *MYCN* and *AURKA* have been illustrated to be present in 40% of t-NEPCs [201], while N-MYC mRNA levels are upregulated in t-NEPCs as compared to CRPCs and primary cancers [200]. Accordingly, clinical responses were observed in a subset of t-NEPC patients treated with the AURKA-inhibitor alisertib [199].

N-MYC overexpression in a genetically engineered mouse model promoted the formation of tumors with NEPC-like characteristics, including low AR signaling and high AKT signaling [200]. Importantly, N-MYC overexpression induced a transcriptional program enriched for PRC2/EZH2 target genes, suggesting that N-MYC may be a critical driver of t-NEPC emergence through epigenetic alterations. Correspondingly, inhibition of EZH2's PRC2-dependent function has been suggested a potential therapy for t-NEPC through epigenetic reprogramming to a less aggressive phenotype [33].

As introduced earlier, it is widely perceived that t-NEPCs (and AI-CRPCs in general), occur as a result of treatment-induced lineage plasticity. Interesting in this regard, the lineage fate of a prostate cancer cell has been implicated to be pre-determined even in localized, treatment-naïve tumors: A diagnosis of aggressive (Grade group 4-5), low PSA (≤2.5 ng/mL) prostate cancer was recently associated with enrichment of neuroendocrine genes and poor outcomes [103]. Corroborative of this notion, surgical castration of the LTL331 patient-derived xenograft (PDX) mouse model from a primary tumor which later recurred to yield t-NEPC led to a predictable t-NEPC emergence *in vivo* [172, 202]. These studies open for the possibility that t-NEPC may occur upon therapeutic challenge of cells that are predisposed to develop lineage plasticity, and may thus involve both clonal selection followed by divergent clonal evolution.

3.6.4. Neuroendocrine transdifferentiation

The concept of NEtD precedes the recent recognition of t-NEPC by several decades [203], and functional preclinical studies on NEtD have laid the groundwork for the current understanding of the t-NEPC subtype. NEtD refers to the reversible process in which cells undergo a switch towards a NE-like phenotype, and is believed to be a central step in the development of t-NEPC [204]. To which extent focal NED resembles t-NEPC at the molecular level is not yet known. NED can be treatment-induced, but also occurs in treatment-naïve tumors (although usually to a low extent). As focal NED is more commonly found in prostate cancer tissue, more knowledge about the link between NED and t-NEPC may have clinical implications.

In patient tumors, NE cells may display endocrine and neuronal characteristics. These include nerve-like dendritic processes extending from the soma (i.e. “open” type NE-cells) [205], but also “closed” type NE-cells [206]. Focal NED is observed histologically by positive staining for one or more NE-markers in 31 to 100% of ADT-naïve primary prostate cancers ([177] and references therein), and is markedly prevalent in metastases [207] and therapy-challenged mCRPCs [169]. However, evidence showing either an indirect or causal relationship between the extent of histologically determined NEtD in localized tumors and t-NEPC development is lacking. Examples of tissue specimens negative and positive for NSE are shown in Figure 3.7 A-B.

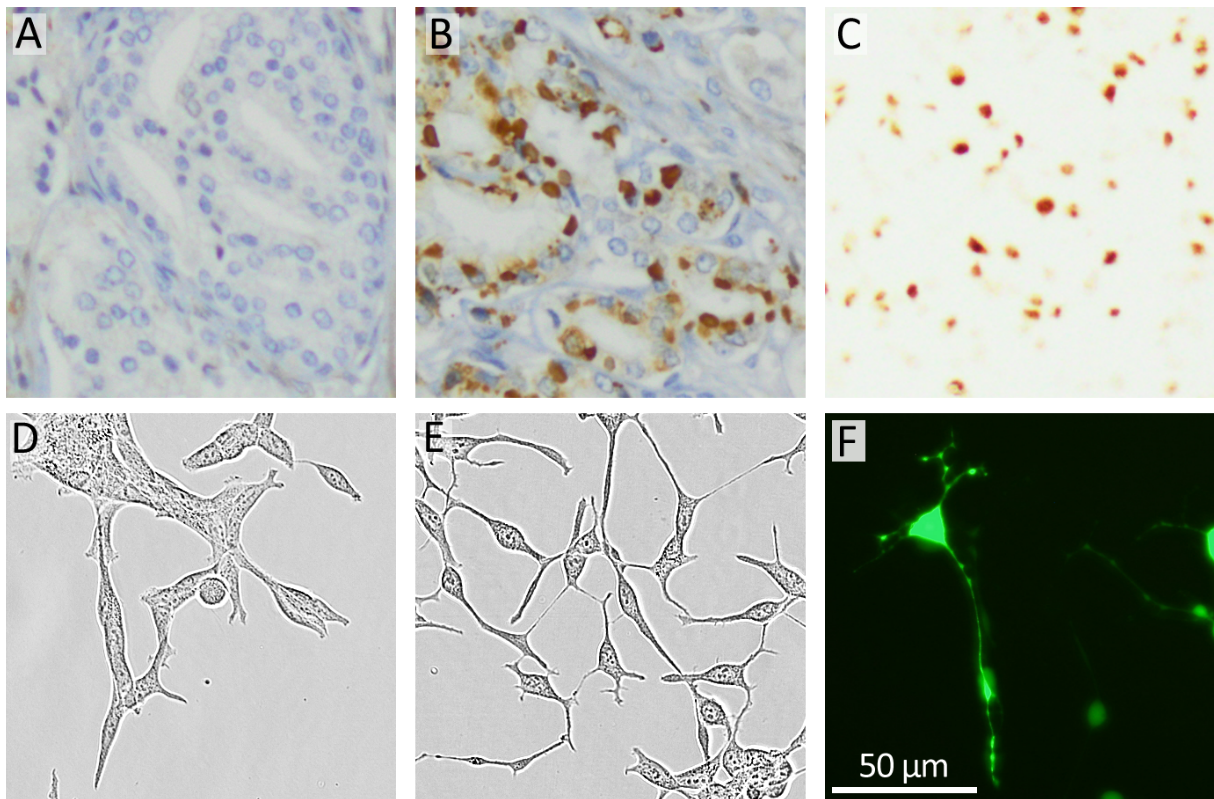


*Figure 3.7. **Neuroendocrine characteristics of clinical prostate cancer specimens and the LNCaP cell line model. A-B.** Immunohistochemical staining of prostate cancer tissue specimens negative (A) and positive (B) for NSE. **C.** Immunohistochemical staining with ENO2/NSE (brown) of paraffin-embedded LNCaP cells pre-treated with 10 µM isoproterenol. **D-E.** LNCaP cells incubated in absence (d) and presence (e) of 10 µM isoproterenol for 24 hours. Images were captured on the IncuCyte FLR platform. D shows differentiated LNCaP cells, while E illustrates pronounced neurite outgrowth with secondary neurite branching and small soma, reminiscent of NE cells. **F.** Fluorescence imaging LNCaP shCtrl cells with ectopic expression of GFP and an ADRB2 overexpression vector displaying a neuronal phenotype.*

There is a lack of consensus on the true lineage of t-NEPC clones. Clues from *in vitro* models seem to favor the luminal-to-neuroendocrine transdifferentiation model. However, in the hormone-sensitive LNCaP cell line, androgen depletion leads to an abundantly documented increase in expression of NE-

markers [196, 208, 209] as shown in Figure 3.7 C. Thus, LNCaP cells in part replicate the clinically observed augmentation of NE features in NED and t-NEPC. The molecular mechanisms driving NE transdifferentiation *in vitro* are not fully understood, but aside from androgen depletion, both ionizing radiation [210], inflammation (i.e. by IL-6) [211, 212]), and stimulation of β-adrenergic receptors (ADRBs) or their downstream signaling pathways [212-215] (Figure 3.7 D-E) all induce NEtD.

Upon NEtD, LNCaP cells progressively develop long neurite outgrowths reminiscent of dendrites and an axon extending from the cell soma (i.e. “open”-type) [213] (Figure 3.7 D-F). This morphological characteristic resembles a subgroup of non-malignant neuroendocrine cells that display dendrite-like processes stretching between benign luminal cells [203], but can also be observed among prostate adenocarcinomas with NED. *In vitro,* the neurites commonly form a complex neural structure and may display secondary and tertiary branching depending on the type, potency, and duration of NEtD-induction. Furthermore, NE-like LNCaP cells display dense cytoplasmic core secretory granules [213] similar to non-malignant NE cells [203]. They produce and secrete a wide variety of molecules believed to contribute to prostate cancer progression [213] and potentially also growth and differentiation of the healthy prostate [177]. Cells proximal to NE-differentiated cells have been shown to exhibit a higher proliferative index, which indicates that NE cells have a paracrine function [216, 217]. Androgen dependence is not necessarily a prerequisite for NEtD *in vitro*, as cAMP-elevating agents can drive NEtD also in androgen-independent C4-2 cells [213].

How well the preclinical observation of NEtD models t-NEPC development is not known to date. Unlike t-NEPCs, NE-transdifferentiated LNCaP cells are initially postmitotic or senescent, but may undergo a proliferative switch upon prolonged androgen deprivation [209, 218-220]. Newly NE-transdifferentiated cells are therefore unlikely to model "true" t-NEPC, but may represent an early transitory state in which the cells are partially committed to t-NEPC which requires further genetic aberrations for the reacquisition of proliferation [221].

3.6.5. The neural trait of prostate cancer

It has been illustrated that cancer cells (including prostate cancer) share genetic characteristics with embryonic neural cells [222]. Gene expression profiling has identified similarities between NE-transdifferentiated LNCaP cells and brain tissue [223]. In the latter study, the “brain profile” was enriched in hormone naïve and castration-resistant metastases, and shared upregulated biological processes included nervous system development, synaptic transmission and neuron differentiation and projections. These findings suggest a progressive acquisition of neural traits during progression.

Transcription of stem cell/progenitor-related genes is tightly regulated by epigenetic restriction during differentiation of human tissues from the embryonic stage to ensure a narrowing of pluripotency [224]. However, upon transformation and oncogenesis, epigenetic reprogramming may re-enable transcription of these silenced genes and hijack developmental processes [18]. Elevated expression of neuronal, EMT and stemness-related genes is observed in t-NEPCs [225, 226], and transcription-activating differential methylation of genes involved in neuronal and stem cell programs has been reported [33]. Both mesenchymal and neural crest cells, the latter which may commit to forming the nervous system, form during early embryonic development. This collectively suggests that prostate cancer cells can hijack these embryonal developmental programs. Hence, reprogramming to a more progenitor-like state seems to occur upon treatment challenge, which in turn can promote lineage plasticity and favor evasion from therapy.

3.7. Biomarkers associated with ADT efficacy

Prognostic biomarkers should be capable of identifying the likelihood of a clinical event or endpoint independent of therapy, while predictive biomarkers should identify patients at baseline who will have a favorable effect of e.g. a novel therapy compared to standard therapy. For the discovery of a predictive biomarker, two patient groups receiving different treatments are thus needed. The predictive biomarker must be able to show differences in the relative efficacy between the two groups based on its level or detection. Thus, identifying an association between a biomarker's level between good- and bad-responders in a uniformly treated patient group does not indicate that the biomarker is predictive, as it may only be prognostic [227]. For this reason, prognostic biomarkers are far more common than predictive biomarkers, and few (if any) predictive biomarkers have been validated for their predictive value in advanced prostate cancer to date [228].

3.7.1. Biomarkers in hormone-naïve prostate cancer

Biopsy studies on HNPC tumors have retrospectively shown that AR protein level is positively associated with duration of response to ADT [229, 230]. In two studies at the Dana-Farber Cancer Institute, biopsies from men with presumed non-localized disease that later underwent ADT were evaluated for clinical and genetic alterations associated with the duration of ADT effect [231, 232]. In their multivariate analyses, homozygous and heterozygous short nucleotide polymorphisms (SNPs) in *CYP19A1*, *HSD3B1,* and *HSD17B4*, all involved in androgen metabolic pathways, were found to be independently associated with shorter time to progression, defined as two rises in PSA over nadir on ADT [231]. In the same study, men with non-metastatic disease experienced longer PFS than men with metastasis at the time of ADT onset. Furthermore, patients who had undergone ADT as part of management of their localized disease showed more rapid progression to CRPC than patients who

received deferred ADT [231]. As introduced earlier, multiple studies show that *AR* gene amplifications, *AR* mutations, and detection of AR-Vs are more frequent in CRPCs than in ADT-naïve tumors [106-108]. These characteristics can be detected both in tissue and bodily fluids [121, 233, 234]. *AR* amplification prompts increased transcription of AR [235], and both amplifications and mutations of the *AR* have been shown to associate with an AR expression score in mCRPC tissue [19]. Hence, these genetic alterations are functionally mirrored by AR transcriptional output. Like alterations in *RB1,* both *TP53* and *AR* associate with short PFS on, or primary resistance to, ARPIs in mCRPC [19, 236, 237], and possibly also ADT responsiveness [238]. Finally, PTEN loss has been associated with shorter OS and PFS on abiraterone [237, 239].

The markers found to associate with ADT-efficacy in the mentioned studies can only be considered prognostic, and further studies with treatment control-groups are needed to evaluate whether they can serve as predictive biomarkers.

3.7.2. Lineage-associated factors

In breast cancer, the PAM50 gene panel is frequently applied to sub-group tumors based on their expression profile, yielding luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-enriched, basal-like and claudin-low subtypes [51]. Subgroup affiliation is strongly associated with prognosis in breast cancer and may predict susceptibility towards endocrine therapies such as tamoxifen. This is exemplified by the lowered treatment responses seen in basal-like and luminal B compared to luminal A breast cancers ([51] and references therein). The lessons learned from breast cancer research has led to the recent application of PAM50 to also partition prostate cancers into molecular subgroups [39]. Here, prostate tumors were divided into either luminal A, luminal B or basal-like lineages, and matched subgroup analyses were performed to investigate the benefit of ADT over no ADT. Whereas both luminal A and basal-like tumors actually had poorer outcomes when treated with ADT than without, luminal B, characterized by the highest expression of luminal markers (e.g. Homeobox protein Nkx3.1 (NKX3-1) and AR signaling genes), showed a clinical benefit of ADT in terms of distant-metastasis free survival (33% for ADT vs. 55% for no ADT).

Zhang and colleagues reported an enrichment of a basal gene signature in both AR-insensitive prostate cancer cell lines, t-NEPCs and *de novo* SCCPs [180], all of which are well known to respond poorly to ADT. In the same study, ADT-treated tumors were more basal-like, whereas conventional ADT-naïve adenocarcinomas were more enriched for a luminal gene signature. Although this study indicates a treatment-related switch from luminal to basal subtypes, metastases were found to be more basal-like than primary tumors, therefore corroborating the notion that lineage plasticity may exist also in a subgroup of ADT-naïve tumors. In the rare cases of *de novo* SCCPs, only the eventual

admixed adenocarcinoma components are believed to respond to ADT, which rationalizes first-line chemotherapy for these patients [240].

3.7.3. Peri-treatment markers of ADT effect

Prostate-specific antigen

PSA levels are monitored during ADT to evaluate treatment effect and biochemical relapse. Having a detectable PSAn during ADT has been associated with a five-fold higher risk of CRPC [241]. Furthermore, PSAn levels higher than 0.2 ng/mL have been shown to associate with poor OS [242]. Patients experiencing a long time to PSAn have improved OS [242-244] and PFS [243], but these studies did not correct for immortal time bias (i.e. that patients with a long time to PSAn necessarily also has a long time to CRPC development). One study investigating the association of time to PSAn following RT with outcome found that the benefit of long time to PSAn was nearly lost after correcting for immortal time bias [245].

Serum testosterone

Upon treatment failure on ADT, it is advised to measure serum testosterone concentrations to evaluate whether unsatisfactory testosterone suppression is achieved or if the disease has indeed progressed to CRPC. In a study performed in Norway in 2006, 10% of men treated with leuprolide (an LHRH agonist) for three months were reported to not achieve castrate serum levels of testosterone [246]. The cut-off used to determine sufficient testosterone suppression was 2.8 nmol/L, however, and using the EAU guidelines (1.7nmol/L) [65] the percentage of men not achieving castration upon LHRH deployment nearly reached 14%.

When serum testosterone levels are insufficiently low, switching to a different LHRH agonist or antagonist may improve suppression to castration levels. A retrospective study showed that the level of serum testosterone and PSA after six months of ADT were associated with mortality [247], underscoring the importance of achieving satisfactory testosterone suppression. To my knowledge, it remains unexplored whether patients with inherent or developed tumoral androgen-hypersensitivity/ responsiveness will respond better upon optimal testosterone suppression.

With the rationale that abrogated or limited AR signaling promotes androgen-independent resistance mechanisms, both supraphysiological testosterone therapy and intermittent ADT have been suggested as potential intervention strategies [248]. Neither treatments are recommended by the EAU [65]. Although promising results were recently reported for supraphysiological testosterone therapy in enzalutamide-resistant PDX models [249], neither treatments have been evaluated for their efficacy towards t-NEPC tumors.

3.8. β_2-adrenergic signaling in prostate cancer

3.8.1. The β_2-adrenergic receptor

The β_2-adrenergic receptor (ADRB2) is a member of the ADRB family of proteins, which also includes β_1- and β_3-adrenergic receptors (ADRB1 and ADRB3, respectively). ADRBs are 7-transmembrane G-protein coupled receptors (GPCRs) that span the plasma membrane of cells. ADRB1s show low mRNA expression levels across all tissues, while ADRB3 is primarily expressed in ovaries [250]. ADRB2s are highly expressed at the mRNA level in the lung, gastrointestinal tract, adipose tissue and prostate [250]. Protein levels of ADRB2 are particularly high in prostate cancers compared to other cancers [250]. ADRB2 is the predominant ADRB subtype in the benign prostate [251] and prostate cancer tissue and cell lines [250, 252, 253]. Whereas ADRB1 mRNA is expressed in prostate cancer, albeit at a low level [250], ADRB3 is not (our unpublished data and [250, 254]). ADRB2 is primarily expressed on epithelial cells in both benign and malignant cells [195, 255], but has also been reported to be expressed on stromal cells [256].

ADRB2 makes out a part of the sympathetic nervous system (SNS). The general role of ADRB2 is to facilitate a rapid stress response and to regulate energy expenditure. The endogenous ligands for ADRBs are the catecholamine adrenergic neurotransmitters epinephrine and norepinephrine. The excitatory ("fight or flight") systemic effects of these hormones include increased heart rate and contraction force (thereby increasing the blood pressure), dilation of the airways, and increased blood supply to smooth muscle cells concomitant with decreased blood supply to the digestive system. Epinephrine and norepinephrine also affect the rate of metabolism through elevating insulin secretion and increasing glycogenolysis and fatty acid catabolism in the liver.

Epinephrine is mainly produced by chromaffin cells of the adrenal medulla [257, 258]. Norepinephrine is also produced by chromaffin cells [258] but is primarily produced in axon terminals in post-ganglionic sympathetic nerves [259]. Both epinephrine and norepinephrine are stored in secretory vesicles alongside ATP and chromogranin A (CHGA) in the mentioned axon terminals [260], which extensively innervate the prostate [3]. From here they are released into the synaptic cleft at which they can bind ADRBs located on post-synaptic neurons or other cell types. Macrophages have also been shown to produce catecholamines, albeit to a minor extent [261], while another study showed that macrophages expressed very low levels of tyrosine hydroxylase (TH; facilitates the rate-limiting step in catecholamine biosynthesis) [262]. Macrophages associated with sympathetic nerves within mouse adipose tissue facilitate the degradation of catecholamines [262]. Specifically, the macrophages expressed the norepinephrine transporter solute carrier family 6 member 2 (SLC6A2)

and monoamine oxidase A (MAOA) which degrades norepinephrine. Importantly, macrophage-driven catecholamine degradation is perceived also to occur in humans [262, 263].

Epinephrine displays a higher affinity towards ADRB2 than norepinephrine, although both molecules show stimulatory effects towards the receptor [264]. Aside from being neuropeptides, epinephrine and norepinephrine also function as circulating hormones and their actions are thus not restricted to tissue innervated by sympathetic nerve fibers.

Upon ligation of a plasma membrane-bound ADRB2, the receptor activates the Gαs guanine nucleotide-binding protein by exchanging guanosine diphosphate (GDP) with guanosine triphosphate (GTP). The coupling of Gαs to ADRB2, in turn, leads to adenylate cyclase (AC) activation (Figure 3.8). AC catalyzes the formation of the second messenger cAMP from ATP. In the canonical ADRB signaling pathway, cAMP activates PKA, which through its ubiquitous kinase activity post-translationally modulates a variety of proteins involved in metabolic processes, differentiation, neurotransmission and cell morphology alterations [265]. Importantly, cAMP-activated PKA can phosphorylate and activate cAMP response element binding protein (CREB), which represents a central TF that regulates transcription of a large number of genes. Aside from CREB, cAMP can activate the Ras-like small GTPase Rap1 [266], which when active may inhibit EMT through regulating epithelial cadherin (E-cadherin) and cell-cell adhesion, ultimately promoting a differentiated epithelial phenotype [267].

The β-adrenergic signaling pathway is implicated in the regulation of steroidogenesis: Isoproterenol, a non-selective epinephrine analogue, as well as epinephrine and norepinephrine were in the 1980s shown to promote testosterone biosynthesis in Leydig cells through promoting cAMP accumulation [268, 269]. Later, in experiments using cultured bovine adrenocortical cells, epinephrine treatment led to an increase in the expression of four Cytochrome P450 genes (including CYP17A1), which conferred increased cortisol levels [270]. Importantly, the Steroidogenic acute regulatory protein (StAR), which controls the rate-limiting step in steroidogenesis, has been reported to be transcriptionally regulated in a cAMP-dependent manner in the gonads and adrenal glands [271]. The StAR gene contains cAMP response elements (CRE) in its promoter region, and it is therefore likely that cAMP-PKA-mediated CREB phosphorylation promotes transcription of StAR. Given the studies illustrating the importance of PKA in promoting AR nuclear translocation and the role of AR in driving transcription of genes within the steroidogenesis pathway, it is perceivable that this pathway also contributes to steroidogenesis in AR-expressing prostatic cells.

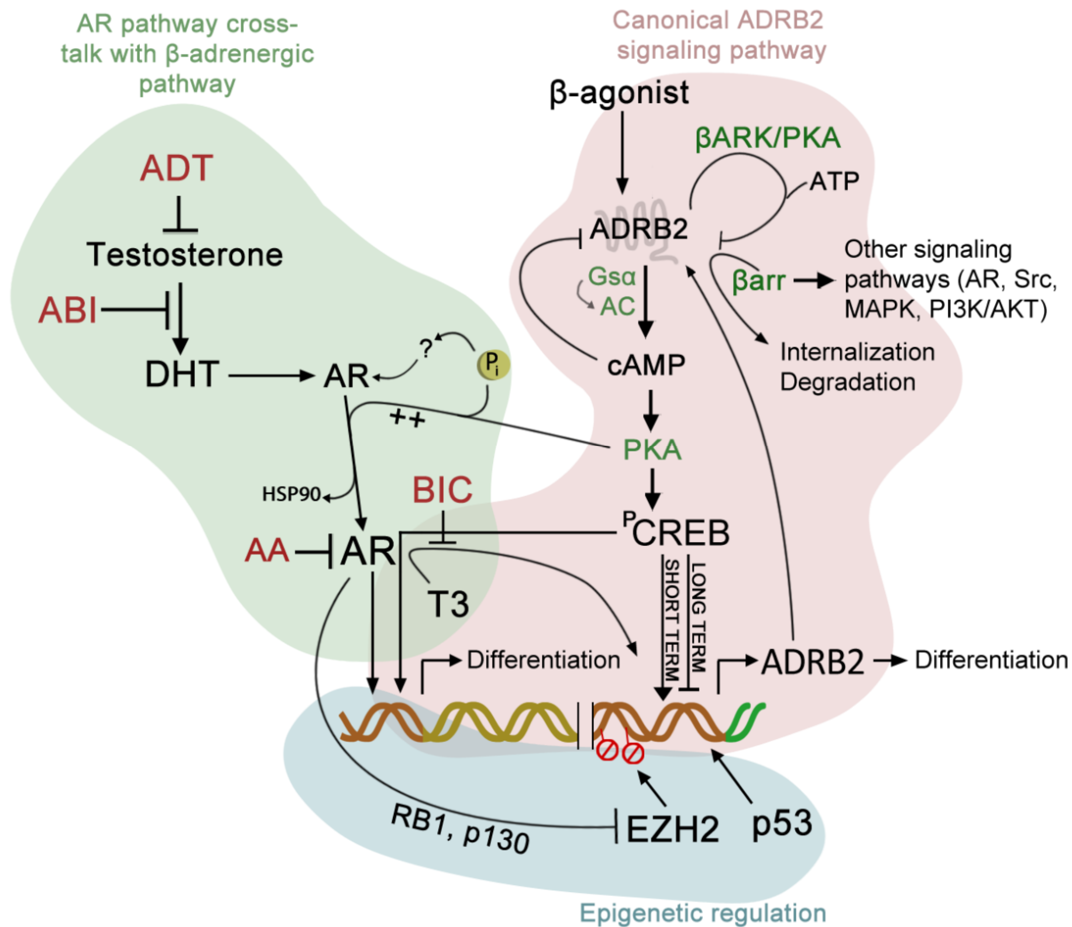


Figure 3.8. ***ADRB2 regulation and its putative cross-talk with the androgen signaling axis.*** *In the canonical ADRB2 signaling pathway, β-agonist binding (e.g. norepinephrine) activates ADRB2, leading to Gαs-dependent activation of adenylate cyclase (AC) which catalyzes cAMP formation from ATP. cAMP activates PKA, which can phosphorylate cAMP response element binding protein (CREB). P-CREB binds to and activates ADRB2 transcription, but during prolonged β-agonist exposure switches towards inhibiting ADRB2 transcription [272, 273]. ADRB2 may be desensitized by phosphorylation from β-adrenergic receptor kinase (βARK) which leads to β-arrestin (βarr) recruitment, receptor internalization and recycling or degradation [274-276]. The AR pathway can cross-talk with the ADRB2 pathway [137]. PKA can facilitate AR nuclear translocation via direct or indirect AR phosphorylation or through phosphorylating HSP90 [154, 155]. Active, nuclear AR can drive expression of e.g. differentiation genes and may drive ADRB2 expression in cooperation with triiodothyronine (T3) or its downstream signaling pathway by a to date unknown mechanism. ADRB2 is also epigenetically regulated, as AR also inhibits EZH2 in an RB1 and p130-dependent manner [193], which inhibits ADRB2 transcription by H3K27 trimethylation [195]. The p53 protein, however, can theoretically activate ADRB2 transcription by binding to its promoter region.*

3.8.2. Regulation of ADRB2 protein and mRNA

The best characterized mechanisms regulating the ADRB2 mRNA level and its protein abundance associates with downstream effects of β-agonist exposure. In this section, post-transcriptional modifications and how these modifications and downstream signaling factors can introduce feedback mechanisms by altering ADRB2 gene transcription are introduced.

Post-transcriptional regulation

Continued agonist exposure is well known to lead to desensitization of GPCRs by negative feedback. Desensitization of ADRB2 is well characterized and involves a rapid and durable PKA or β-adrenergic receptor kinase (βARKs, also known as GRKs and ADRBKs)-mediated phosphorylation of the receptor within seconds or minutes after agonist exposure [274-276]. βARKs facilitate β-arrestin recruitment and binding to phosphorylated ADRB2s. This, in turn, promotes desensitization and receptor internalization and subsequent recycling or endocytosis [277]. β-arrestins are themselves signaling transducers [278] acting on the PI3K/AKT and mitogen-activated protein kinase (MAPK) pathways [279, 280] among others. Hence, the desensitization of the β-adrenergic receptor links the canonical ADRB2 pathway with other signaling pathways.

Whereas PKA-mediated desensitization is dependent on elevated cAMP and consequently increased PKA activity, βARK phosphorylates ADRB2 when the receptor holds an active sterical conformation. Collins *et al.* reported that prolonged exposure to epinephrine or a dibutyryl analogue derived from cAMP reduced ADRB2 protein levels by 80% and by 55%, respectively, in DDT1MF-2 cells [281]. The reduction in ADRB2 protein level was accompanied by loss of AC activity. Our group showed that isoproterenol exposure led to a transient increase in cAMP levels in prostate cancer cell lines [255]. After 15 minutes of exposure to isoproterenol, cAMP levels were in fact lowered than before stimulation, illustrating that ADRB2 desensitization rapidly occurs upon exposure. In the same study, incubation of LNCaP cells in either androgen-depleted (charcoal-stripped FCS; CSS) medium or androgen-containing serum supplemented with bicalutamide led to lowering of ADRB2 protein levels. Triiodothyronine (T3) was found to rescue CSS-mediated ADRB2 downregulation, but only in the absence of bicalutamide, indicating that T3-mediated rescue was dependent on unobstructed AR signaling.

Transcriptional regulation

Multiple studies in different model systems have shown that ADRB2 transcript levels drop upon prolonged β-agonist exposure [272, 273, 281, 282]. In two studies on S49 mouse lymphoma and MF-2 hamster vas deferens cells, Hadcock and colleagues showed that prolonged agonist exposure [272, 273] and activation of adenylate cyclase by forskolin [273] led to ADRB2 mRNA downregulation.

Similar effects have been reported in rat C6 glioma cells and DDT1MF-2 cells [281, 282]. Conversely, the Lefkowitz laboratory showed that short-term β-adrenergic agonist exposure (30 min) led to elevated ADRB2 mRNA levels [281, 283], possibly as a means to compensate for receptor degradation upon desensitization. The ADRB2 promoter was reported to contain cAMP response elements (CREs) [283]. CREB, activated by cAMP-PKA downstream of the β_2-adrenergic receptor, binds to CRE elements in gene promoters. If the human ADRB2 promoter indeed contains active CRE-elements, this would represent positive feedback autoregulation of ADRB2, which may function as a mediator of ADRB2 protein levels during high receptor turn-over upon short-term agonist-exposure. As levels of epinephrine and norepinephrine rapidly spike upon stress, but also rapidly return to baseline after stress cessation, ADRB2 autoregulation should theoretically prevent maintained loss of ADRB2 protein due to desensitization-mediated endocytosis. During long-term exposure, however, downregulation of ADRB2 can prevent prolonged, hyperactive and potentially pathogenic stress responses [284].

Aside from being regulated by feedback mechanisms, ADRB2 can also be epigenetically regulated: In a study by Mohn *et al.,* lineage-specific Polycomb targets were investigated for their differential methylation and expression during differentiation towards neurons [18]. As stem cells differentiate to lineage-committed progenitors and further to terminally differentiated neurons, the ADRB2 promoter was progressively trimethylated at H3K27s, leading to ADRB2 silencing [18]. The findings suggested that epigenetic silencing of ADRB2 may be a requisite event during reprogramming from stem cells to terminally differentiated neurons.

The EZH2-PRC2 complex catalyzes the deposition of repressive marks (H3K27me3) at the ADRB2 promoter through its PRC2-dependent function, consequently silencing ADRB2 [195]. Due to the oncogenic role of EZH2, ADRB2 has been suggested to represent a tumor suppressor [285]. In support of this, ERG, which regulates EZH2, also silences ADRB2 independently of EZH2 [286, 287]. Furthermore, ERG overexpression in RWPE-1 cells has been shown to reduce expression of Transcription factor SOX9 (SOX-9), vimentin, and ADRB2, and upregulated E2F Transcription factor 1 (E2F1) [286]. Overexpression of ETS variant 1 (ETV1), however, leads to upregulation of SOX-9, vimentin and ADRB2 mRNA levels while reducing E2F1 expression, indicating opposite effects of these two ETS TFs. Given the unlikely coincidental co-regulatory landscape of ADRB2 with genes well-established to be important in AR signaling, EMT, cell cycle and upon ETS-related gene fusion events, it may well be that ADRB2 plays functional roles in these processes as well.

Figure 3.9 shows results from an integration of multiple chromatin immunoprecipitation sequencing (ChIP-seq) analyses performed on multiple cell line models and prostate cancer tissue [287-292]. The

analyses identified TF binding sites for CCCTC-binding factor (CTCF), FOXA1, ETV1, ERG and MYC in the ADRB2 promoter, as well as AR, HOXB13 and GR binding sites in what appears to be enhancer regions up- and downstream of the ADRB2 exon. Several TFs have also been predicted *in silico* to be present in the ADRB2 promoter, including binding sites for RE1 Silencing Transcription Factor (REST), E2F1, FOXA2, RB1, p53 and GR (ConTra v3, from ADRB2 5'UTR - 500bp upstream [206], www.Genecards.org, Qiagen).

Of note, REST has been reported to repress neuronal genes and prevent NEtD [293] and E2F1-activated transcription of EZH2 in bladder and prostate cancer [294, 295]. A positive correlation between REST and EZH2 has been documented in prostate cancer organoids [188]. Furthermore, FOXA1 may repress NEtD in LNCaP cells [296], and the proto-oncogene MYC is increased in most advanced prostate cancers and mCRPCs [297]. Thus, multiple TFs shown to be aberrant may act on the *ADRB2* promoter.

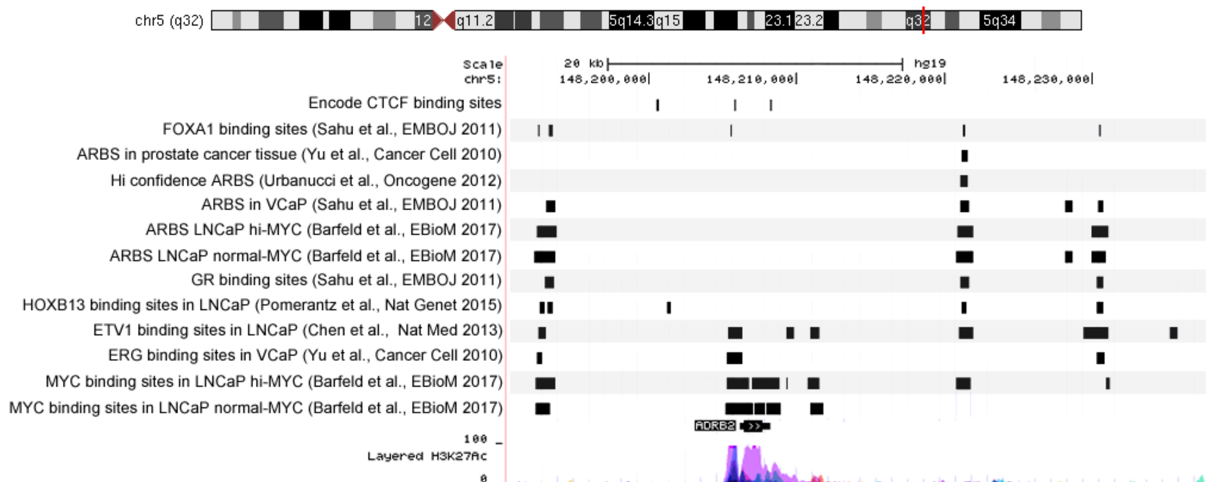


Figure 3.9. ***ChIP-seq analyses of binding sites in proximity to the ADRB2* gene. *A.*** *The intronless ADRB2 gene (chromosome 5, q32) spans 2042 base pairs. The ADRB2 exon is shown at the lower part of the plot, aligned layered H3K27Ac (indicates active transcription) and various TF binding sites (FOXA1, AR (ARBS), GR, HOXB13, ETV1, ERG, and MYC) identified in prostate cancer tissue and untreated and treated cell lines. The black boxes indicate significant TF binding aligned to the hg19 build. The plot was generated using custom uploaded tracks to the UCSC Genome Browser [298].*

Hormonal regulation of ADRB2

An androgen-based regulation of ADRB2 protein and mRNA level is also plausible: Marchetti and colleagues showed in 1988 that rat prostate ADRB2 protein levels dropped within ten days of castration, and that testosterone increased the receptor numbers [299]. Twenty years later our group showed that ADRB2 mRNA levels were lowered in LNCaP and LNCaP-derived C4-2 cells incubated in androgen-depleted compared to androgen-containing medium [255]. In the same study,

T3 rescued ADRB2 transcription in both cell line models, but only in absence of bicalutamide, which by itself decreased ADRB2 protein levels nearly as much as androgen depletion. The downregulation of ADRB2 following androgen depletion seems to be maintained upon prolonged treatment: In the LNCaP-derived LNCaP-Rf cells, which have regained proliferation following long-term androgen depletion, ADRB2 mRNA levels were hundred-fold lower than in its parental LNCaP model [255]. In support of this, NCI-H660 and androgen-deprived LNCaP cells expressed nearly diminished ADRB2 mRNA levels measured by RNA-sequencing (RNA-seq) [254] and microarray [300] (accessible under GSE25183 and portals.broadinstitute.org/ccle, respectively).

Although androgen depletion induces a predictable lowering of ADRB2 mRNA and protein, there is a lack of evidence showing a positive regulation upon stimulation of AR. Although two studies with appended transcriptomic data indicate a transient upregulation of ADRB2 upon R1881 (a synthetic androgen)-stimulation of LNCaP cells [301, 302], another data set does not show ADRB2 upregulation in LNCaP and VCaP cells upon AR stimulation [303]. Given that the *ADRB2* gene promoter probably lacks ARBSs in prostate cancer cell lines, but contains putative ARBSs in what may be enhancer elements further upstream, androgenic regulation of ADRB2 cannot be ruled out. An explanation for the strong androgen depletion-induced downregulation of ADRB2 yet lack of androgen-mediated rescue remains to be elucidated.

3.8.3. ADRB2 in prostate cancer

In the rat prostate, catecholamines promote differentiation [304]. Accordingly, Yu *et al.* showed that knockdown of ADRB2 in benign prostate RWPE-1 promoted EMT and loss of luminal differentiation [195]. These studies collectively point towards a differentiation-promoting role of ADRB2 in the healthy prostate (Figure 3.8). Given the mentioned relationship between β-adrenergic signaling and steroidogenesis, a functional overlap between the ADRB2 and AR signaling axes is plausible.

Although studies indicating a role of ADRB2 in cancer were reported nearly 50 years ago [206], the receptor has gained increasing attention and traction after the more recent studies in various cancer types illustrating the sympathetic nervous system as a critical factor in oncogenesis and cancer progression [305, 306].

Sympathetic nerves are found to innervate nearly all tissues in humans. It is therefore not surprising that these nerves are also present in malignant lesions. Multiple studies suggest that the nerves are not only bystanders during neoplasia and cancer progression, however. Cancer cells are known to release neurotrophic growth factors such as nerve growth factor (NGF) that stimulate and direct nerves to the tumor and the tumor microenvironment (TME) in a process termed axonogenesis [307]. In a landmark study by Magnon *et al.,* adrenergic nerve fibers were found to innervate tissue

adjacent to primary prostate tumors, and high nerve densities were associated with an elevated tumor proliferation index [308]. Furthermore, adrenergic nerves also correlated with preoperative PSA-levels and associated with BCR and metastasis. Axonogenesis in the prostate TME is associated with aggressive disease and BCR [309, 310], and has been shown to correlate with proliferation and expression of proteins involved in pro-survival pathways [310]. A similar association between nerves and cancer exists in breast cancer, where higher nerve densities were reported in high-grade than in low-grade breast cancers [311]. Hence, tumoral innervation is not only coincidental but represents a feature that can be exploited by cancer cells, presumably through hijacking the developmental program of axonogenesis.

The oncogenic properties of nerves have been hypothesized to be linked to their importance in wound repair (comment by D. Rowley in [312]). Like tissue damage, cancerous lesions promote inflammation and engage appropriate immune responses. Nerve input in the regenerative growth zone is essential for limb regeneration following amputation in amphibians, where stem/progenitor cells have an essential function (reviewed in [313]). During this process, neurogenesis/axonogenesis into the wounded zone where the stem/progenitor cells are located (termed the blastema) facilitates the regenerative process. β_2-adrenergic receptor activation reportedly promoted skin wound healing in mammalian model systems [314] and was found to be functionally involved in muscle regeneration following injury [315]. Tumors share multiple characteristics with the blastema, such as stem/progenitor cell dependence, which supports the importance of nerves in cancer and a potential stimulatory effect of neurotransmitters such as norepinephrine on stem/progenitor cells [313].

As introduced earlier, ADRB2 is a crucial mediator of catecholamine action, and it is expressed both on prostate cancer cells, endothelial cells and stromal cells that collectively make out part of the TME. The concept of a sympathetic nerve-cancer cell cross-talk, whereby cancer cells attract nerves to facilitate catecholamine exposure on ADRB2s to promote cancer initiation, metastasis and potentially PNI, may have therapeutic implications [316]. In the work by Magnon *et al.,* denervation of sympathetic and parasympathetic nerves upstream of mouse prostates abrogated prostate cancer initiation and growth [308] (Figure 3.10). Importantly, chemical castration nearly completely abolished metastatic spread in orthotopic PC-3 xenografts. These tumors grew slower in ADRB2 knockout mice (ADRB2$^{-/-}$) than in wild-type mice (both with intact sympathetic nerves), indicating a functional role of ADRB2s expressed in the TME. Supporting the study by Magnon, both bilateral denervation and botox treatment (a derivative of the nerve poison botulinum toxin) was more recently shown to nearly completely abrogate orthotopic VCaP mouse and rat xenograft tumor growth [317]. Unilateral botox injections led to lowered tumor growth on the botox-treated sides of the mouse prostates as compared to the saline-treated sides. As a proof-of-concept, a small clinical

trial on four men with localized disease illustrated that unilateral botox injections led to decreased nerve densities, elevated apoptosis, and a distinct transcriptional profile in the tumor and normal tissue as compared to the saline-treated sides. Interestingly, the transcriptional expression profiles of Botox-treated prostate tumors resembled that of a prostate cancer patient with spinal cord injury [317].

Whether the oncogenic properties of cancer-innervated nerve fibers primarily act on the TME, the cancer cells directly, or both, remains an unanswered question. Studies on an ADRB2$^{-/-}$/ADRB3$^{-/-}$ orthotopic PC-3 xenograft model indicated that the nerve fibers elicit their effect on ADRBs in the TME [308]. Later it was shown that catecholamine released from sympathetic nerves infiltrating prostate tumors induced a growth-promoting angio-metabolic switch mediated by ADRB2 present on endothelial cells (Figure 3.10) [318]. It is plausible, however, that ADRB2s present on the tumor cells are also important for the receptor's oncogenic properties, as illustrated in Figure 3.10: In the study by Yu, short hairpin RNA (shRNA)-mediated knockdown of ADRB2 was sufficient to drive malignant transformation of immortalized benign RWPE-1 prostate epithelial cells [195]. Isoproterenol significantly inhibited prostate cancer DU145 xenograft tumor growth, and EZH2 knockdown in the same cell line inhibited tumor formation, collectively illustrating that cancer cell membrane-bound ADRB2 was associated with differentiation and low tumorigenic potential.

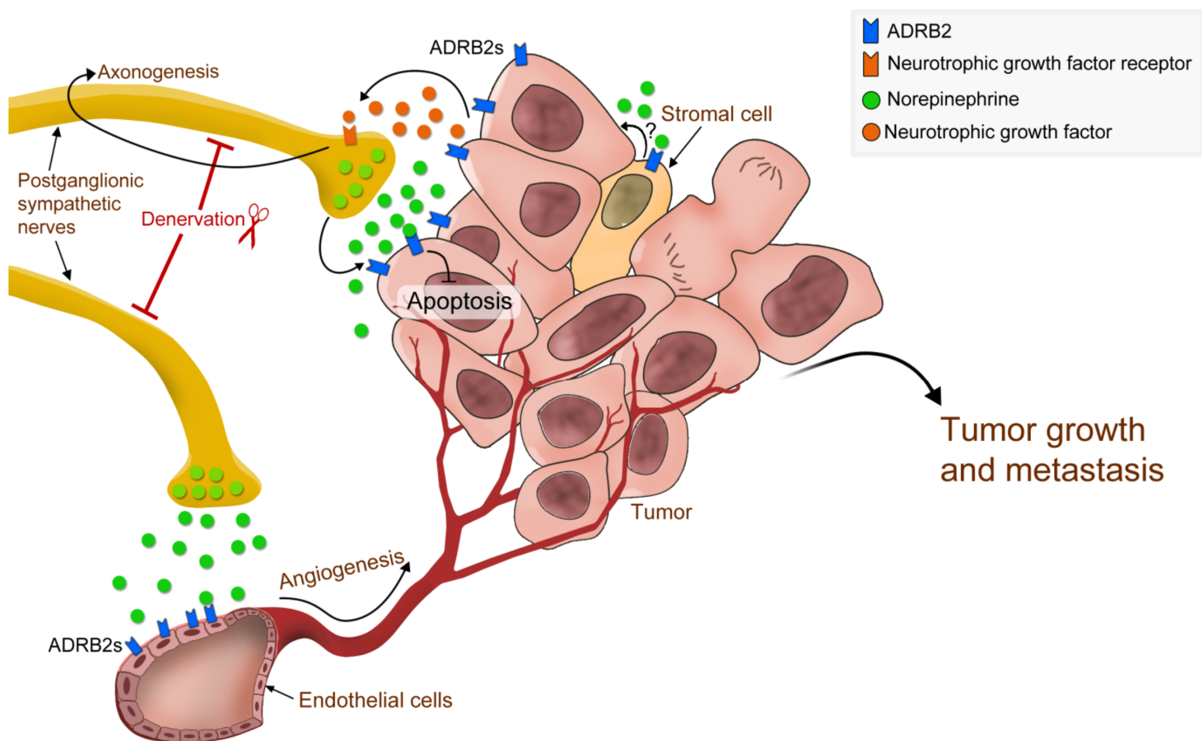


*Figure 3.10. **The sympathetic nervous system as a part of the tumor microenvironment.** Norepinephrine secreted by sympathetic nerves innervating the TME stimulates ADRB2s on cancer cells [195] and inhibits*

apoptosis [284, 319]. Neurotrophic growth factors secreted by cancer cells stimulate axonogenesis [307], potentiating the sympathetic tone on the tumor in what may be a feed-forward loop. Norepinephrine can also stimulate ADRB2s situated on stromal and endothelial cells in the TME [308, 318, 320]. Stimulation of endothelial cells promotes angiogenesis and tumor vascularization. Given the growth- and metastasis-inhibiting effects of denervation, sympathetic stimulation may promote these features.

Yu's study can be considered controversial, as many studies have indicated that β-adrenergic signaling has a cancer-promoting effect [156, 320-324]. The Entschladen group showed using *in vitro* monoculture experiments that norepinephrine promoted migration of prostate [321, 322], breast [322, 323] and colon cancer cells [324]. In an ovarian cancer mouse model, behavioral stress was shown to elevate catecholamine levels and accelerate tumor growth by directly activating ADRB2s on the tumor cell membranes [320]. Catecholamines increased tumor vascularization and Vascular endothelial growth factor (VEGF) expression, which indicates that stimulation of ADRB2s may promote angiogenesis through acting both on the tumor cells and on the TME [318, 320, 325]. In two publications from the Kulik group [284, 319], catecholamine action on ADRB2s elicited anti-apoptotic effects on prostate cancer cells cultured *in vitro* and in subcutaneous xenograft models. The antiapoptotic effect was shown to be mediated via ADRB2-cAMP-PKA-mediated inactivation of the pro-apoptotic Bcl-2-associated death promoter (BAD) protein [319]. In *PTEN*-deficient mice with constitutively active PI3K/AKT signaling (which inhibits apoptosis), pro-apoptotic PI3K-inhibition was abrogated by catecholamine-elevating immobilization stress [284]. Again, the effect was dependent on PKA-mediated BAD phosphorylation, and a specific ADRB2 antagonist inhibited the antiapoptotic effect of catecholamine exposure.

Kasbohm and colleagues showed that ADRB2 activation led to elevated AR activity in prostate cancer cells [156]. Importantly, ADRB2 activation potentiated DHT-mediated stimulation of the AR. The cAMP-activated PKA phosphorylated the AR, thus illustrating a permissive role of PKA in nuclear translocation of AR. Considering the androgen-mediated regulation of ADRB2 (introduced earlier), this study illustrated the presence of reciprocal regulatory mechanisms connecting the β_2-adrenergic and AR signaling pathways. Supporting this putative complementarity, DHT-mediated AR activation has been shown to lead to an increase in cAMP levels [156], and Wang *et al.* reported that several target genes of the β-adrenergic and androgen signaling pathways are shared [326]. In the same study, commonly upregulated genes upon forskolin and R1881-treatment included HMG CoA reductase (HMGCR), which catalyzes a rate-limiting step in cholesterol biosynthesis, and KLK3 [46]. Meanwhile, e.g. Neuron Navigator 1 (NAV1), involved in axon guidance and neuronal development, was downregulated upon exposure to both agents.

ADRB2 and neuroendocrine transdifferentiation

As briefly introduced earlier, stimulation of ADRB2, the predominant ADRB subtype in prostate cancer, or its downstream signaling pathway, induces NEtD [212-215]. The underlying mechanisms remain largely unknown, however. As NEtD induces marked morphological changes in LNCaP cells, cytoskeletal rearrangements are likely to occur during neurite outgrowth and rounding of cell soma. Neurite outgrowth in LNCaP cells may be induced by PKA/p21-activated kinase 4 (PAK4)-mediated Ras Homolog Family Member A (RhoA)/Rho-associated coiled-coil kinase-associated kinase (ROCK) pathway inhibition which leads to cytoskeletal rearrangements [325, 327, 328]. In one study, PKA-phosphorylated PAK4 was essential for cAMP-induced NEtD, measured by increased neurite outgrowth and NSE expression [328]. Another study reported that activation of guanine nucleotide exchange protein activated by adenylate cyclase (EPAC) inhibited both cytoskeletal integrity, MAPK signaling, and RhoA activation in PC-3 and DU145 cells [329].

Interestingly, Sang and colleagues reported a higher degree of phosphorylated CREB (i.e. active CREB) in NEPC-like NE1.3 and NCI-H660 cells than in LNCaP and VCaP cells which have an adenocarcinoma lineage [330]. In this study, CREB drove transcription of GRK3, and stable GRK3 knockdown inhibited CREB-induced NEtD in LNCaP cells. Whether GRK3 led to β-arrestin recruitment and ADRB2 desensitization was not addressed. One study has however shown that overexpression of GRKs, including GRK3, leads to increased β-arrestin recruitment to ADRB2 [331].

Associations between ADRB2, prostate cancer progression and clinical outcomes

Aside from its functional involvement in prostate cancer progression, ADRB2 has been associated with clinical outcomes in multiple cancer types. High ADRB2 levels have been associated with poor OS in malignant melanoma [332], gastric [333], liver [334] and pancreatic cancer [335]. Conversely, studies on squamous cell carcinoma [336] and breast cancer [337] have indicated that high ADRB2 levels associate with improved OS.

In localized prostate cancer tissue, low ADRB2 immunohistochemical staining intensity has been associated with rapid BCR post RP [195]. Interestingly, our group and others have shown that incidental beta-blocker use (pharmacological inhibition of ADRBs) is associated with improved prostate cancer-specific survival [338-340]. We have also shown that ADRB2 mRNA levels are downregulated in tumors treated with neoadjuvant ADT [255], and Yu showed that ADRB2 mRNA was downregulated in metastases as compared to primary tumors [195]. Collectively these data point towards differential regulation of ADRB2 transcription during disease progression, which may indicate a functional involvement of ADRB2 in therapy resistance.

4. Aims

There is a predictable emergence of castration resistance in prostate cancer patients undergoing ADT. Despite the extensive efforts laid into developing treatment strategies for patients relapsing upon ADT, CRPC remains a lethal disease. Through acquiring a better biological understanding of the mechanisms facilitating resistance to ADT one may identify actionable targets to prevent or manage CRPC. Deciphering molecular characteristics associated with lineage plasticity and divergent evolution upon targeted treatment will aid in the tailoring treatment regimens and thereby improve personalized medicine.

Upregulation of intratumoral steroidogenesis is a putative mechanism for prostate cancer cells to maintain androgen signaling and thereby circumvent ADT. Based on our preliminary findings and the association between ADRB2, AR signaling, and steroidogenesis, we wanted to:

- Elucidate the role of ADRB2 as a prognostic biomarker in advanced prostate cancer and its mechanistic involvement in ADT-resistance development (Paper I and III)

The recent increase in t-NEPC has prompted extensive efforts to understand the biology underlying this lethal prostate cancer subtype. Stimulation of ADRB2 or its downstream signaling cascade is well known to induce neuroendocrine transdifferentiation (NEtD). NEtD is perceived to be a necessary step in the emergence of t-NEPC. As ADRB2 could thus represent a druggable target in t-NEPCs, we aimed to:

- Explore whether ADRB2 is functionally involved in androgen depletion-induced NEtD, and whether differential ADRB2 expression could associate with lineage fate following androgen-targeted therapies (Paper II)

Despite the generally favorable prognoses of patients undergoing radical prostatectomy with curative intent, around 5% of these patients are believed to develop advanced prostate cancer with ADT as the preferred first-line treatment. Identifying molecular markers prognostic of resistance to androgen-targeted therapies at the time of surgery will lay the grounds for predictive biomarker studies that can improve treatment decision for these patients. Towards this end, we sought to:

- *Investigate whether transcriptional signatures in hormone-naïve radically resected tumor specimens associate with resistance towards androgen-targeted therapies (Paper III)*

5. Summary of papers

5.1. Paper I: Low β_2-adrenergic receptor level may promote development of castration-resistant prostate cancer and altered steroid metabolism

The androgen receptor (AR) and its associated signaling pathway remain pivotal in the clinical management of locally advanced and metastatic prostate cancer (PCa). Indeed, androgen deprivation therapy (ADT), which lowers systemic testosterone levels, invokes a clinical response in most patients [16]. Reduced bioavailability for testosterone selectively targets androgen-dependent cancer cells, but resistance to ADT is routinely observed. The disease then manifests as castration-resistant PCa (CRPC), which is characterized by disease progression despite castration testosterone levels [65]. Patients respond differentially to ADT, and while many patients experience symptomatic relief and lengthy progression-free survival, some patients seem inherently resistant and rapidly progress.

The androgen metabolic pathways are commonly aberrant in CRPCs, and represent a putative intracrine adaptation mechanism to sustain AR signaling during androgen deprivation. The β_2-adrenergic receptor (ADRB2) has been implicated in CRPC development, as stimulation of ADRB2, like DHT, led to a dose-dependent increase in ARE-regulated reporter activity [156]. The downstream effectors of ADRB2, specifically cyclic AMP (cAMP) and cAMP-dependent protein kinase A (PKA), have been shown to promote the activity of the AR [137] and to control the transcription of steroidogenic enzymes such as CYP17 in adrenocortical cells [341, 342]. These findings led us to investigate whether differential ADRB2 expression in the androgen-sensitive and dependent PCa LNCaP cell line model would impact the AR signaling pathway and thereby CRPC development.

We analyzed ADRB2 staining intensity in transurethrally resected (TUR-P) PCa tissue specimens. We identified an inverse association between ADRB2 staining intensity and time to the development of CRPC following ADT, independently of age and Gleason score. Using short hairpin RNAs, we generated LNCaP ADRB2 knockdown clones (shADRB2-1 and -2) and a control cell line (scrambled; shCtrl). The shADRB2 cell lines expressed lowered ADRB2 mRNA and protein levels than shCtrl cells. When xenografted into NOD-SCID gamma mice, shADRB2-2 tumors grew more rapidly than shCtrl tumors following surgical castration. Transcriptomic profiling of the shADRB2 cells revealed a sharp decline in mRNA levels of multiple members of the UDP-glucuronosyltransferase 2B gene family (UGT2B) compared to shCtrl, which manifested in lowered protein expression of UGT2B15 and UGT2B17. Concomitant with lowered UGT2B15 and UGT2B17 expression, the shADRB2 cells produced lowered amounts of glucuronidated androgen metabolites both *in vitro* and *in vivo*. Upon stimulation with glucuronidable DHT, but not with non-glucuronidable R1881, shADRB2 cells

displayed higher androgen responsiveness under androgen-depleted conditions than shCtrl cells, as measured by the luciferase-driven activity of multiple androgen-responsive reporters and PSA secretion *in vitro*. Lowered glucuronidation in shADRB2 cells was accompanied by elevated testosterone levels, and UGT2B inhibition led to notable increases in testosterone levels in shCtrl cells. Finally, immunohistochemical analyses revealed positive correlations between ADRB2 and UGT2B15 and UGT2B17 staining in two independent cohorts, and like ADRB2, low UGT2B15 staining intensity was associated with CRPC development.

Taken together, the findings presented show an inverse association between ADRB2 and CRPC development that has not been previously reported. This suggested that ADRB2 is a prognostic biomarker for ADT efficacy. The preclinical data indicate that androgen elimination by glucuronidation is regulated by ADRB2 in PCa cells, and that depletion of ADRB2 facilitates the emergence of CRPC through promoting re-emergence of androgen signaling by increasing androgen levels in an otherwise androgen-deprived milieu.

5.2. Paper II: The β_2-adrenergic receptor is a molecular switch for neuroendocrine transdifferentiation of prostate cancer cells

In paper I we reported that prostate cancer tumors with low levels of ADRB2 more rapidly develop CRPC adenocarcinoma, possibly through better retaining bioavailable androgens as was found in our *in vitro* model system. The majority of castration-resistant prostate cancers (CRPCs) are adenocarcinomas and are characterized by aberrations driving androgen receptor (AR) signaling, including altered steroid metabolism. However, there has been a recent surge in the prevalence of CRPCs displaying visceral metastatic spread, disproportionately low PSA and neuroendocrine differentiation, commonly termed treatment-related NEPC (t-NEPC). The increased emergence of t-NEPC tumors has prompted investigations into the underlying mechanisms driving their development, and their increased prevalence has coincided with a more widespread implementation of drugs targeting the androgen signaling axis. These drugs are believed to promote lineage plasticity and lowered reliance on AR signaling, thus enabling therapeutic resistance. Treatment-related NEPCs are perceived to clonally evolve from CRPC adenocarcinomas, suggesting that lineage plasticity may be a general feature of at least a subset of CRPCs.

Neuroendocrine transdifferentiation, a process in which cells reversibly reprogram to a neuroendocrine-like state characterized by neural-like morphological changes and elevated neuroendocrine (NE) marker expression, is believed to be a prerequisite step in the development of t-NEPC. Stimulation of β_2-adrenergic receptors (ADRB2s) is well known to induce NEtD in various PCa cell line models, and we hypothesized that ADRB2 could be a druggable target to treat t-NEPCs.

To this end, we investigated the effect of ADRB2 depletion on hormone-sensitive PCa LNCaP cells put under treatment challenge. Stable knockdown of ADRB2 reduced expression of genes annotated *neuron differentiation,* and also impeded both neurite outgrowth and NE-marker expression following androgen depletion. Stimulation of ADRB2 using a β-agonist induced neurite outgrowth and this effect was lost upon sufficient ADRB2 depletion or concomitant β-blocker administration. Low-ADRB2 cells had higher canonical Wnt activity, and inhibition of GSK3α/β-mediated β-catenin activation reduced NE-like characteristics. Transient overexpression of ADRB2 markedly increased neurite outgrowth, but not NE-marker expression levels, in both hormone-sensitive and -insensitive PCa cell lines. In clinical material, ADRB2 protein levels associated with luminal differentiation and low Gleason grades, was upregulated in metastases, and was progressively downregulated in tumors resistant to AR-targeted therapies and in t-NEPCs. When grown in mice, high-ADRB2 tumors had lower tumor latencies and were more determined than low-ADRB2 tumors to NE-transdifferentiate.

Collectively, this study illustrates that ADRB2 is functionally involved in NEtD. While high ADRB2 levels determine a NE lineage fate upon androgen depletion, its expression is progressively lost during dedifferentiation and therapy resistance, particularly in t-NEPCs. Low-ADRB2 tumors may be more determined to a luminal lineage and the development of CRPC adenocarcinoma through re-activation of the AR signaling axis, as shown in Paper I. As ADRB2 is a target of β-blockers, which are off-patent drugs with safe profiles, β-blockers may be repurposed to prevent high-ADRB2 tumor cells from undergoing treatment pressure-induced lineage transdifferentiation and reprogramming towards a neuroendocrine lineage. Maintaining androgen responses will enable more patients to experience a clinical benefit from androgen-targeted therapies rather than develop lethal t-NEPC.

5.3. Paper III: Bicalutamide treatment in hormone-naïve prostate cancer associates with cross-resistance to androgen deprivation therapy

Due to the commonality of androgen dependence of prostate cancer tumors, ADT confers a clinical response in most patients. The duration of the response varies greatly, however, and some tumors display inherent ADT resistance, illustrated by a lack of PSA-response. Little is known however about which patients will respond and not, and whether salvage treatments associate with ADT effect. Yet, ADT remains the cornerstone and first-line treatment option for advanced and metastatic prostate cancer. In this study, we integrated clinical and transcriptomic features, as well as cell line experiments, to elucidate whether primary, hormone-naïve tumor characteristics associated with response to androgen-targeted therapy. Concomitantly, we sought to verify the negative association between ADRB2 staining and CRPC emergence using transcriptomic profiling.

Through a screen of 2500 patients radically operated for prostate cancer between 1994 and 2015 we identified 71 men treated for radiation- and hormone-naïve PCa who later underwent ADT and who had complete clinical and pathological records. We collected baseline demographics, tumor-related characteristics and clinical follow-up data for the included patients, including progression-free survival (PFS) times on salvage bicalutamide and ADT. Extracted RNA from 37 patients was gene expression profiled by cDNA microarray.

We found that salvage therapy, including salvage RT (sRT) and salvage bicalutamide was associated with delayed onset of ADT but expedited CRPC development. Patients experiencing short PFS on bicalutamide were at higher risk for rapid CRPC, and this association was independent of tumor-related characteristics. Tumors from non-salvage treated patients had higher pre-operative PSA levels and less differentiated tumors. Gene expression profiling showed that the β_2-adrenergic receptor (ADRB2) was among the differentially expressed genes with the highest fold-change between patients not responding and responding to ADT. High expression of ADRB2 was associated with rapid CRPC development. This association was however dependent on bicalutamide administration. Further analyses revealed that low ADRB2 expression levels associated with treatment failure on bicalutamide and downregulation of androgen-responsive and androgen receptor (AR) signaling-related genes. These genes were enriched in tumors from patients later treated with salvage bicalutamide and accordingly also tumors responding poorly to ADT. Stimulation of PCa cell lines using the β-agonist isoproterenol led to activation of the AR. This effect was dependent on functional AR signaling and AREs, and isoproterenol potentiated R1881-driven AR activity. Finally, antiandrogens abrogated isoproterenol-mediated increases in AR activity.

The presented findings illustrate that while salvage bicalutamide delayed the onset of ADT, it may have imposed priming of AR/androgen independence and thus ADT-resistance. We have previously shown that low ADRB2 expression associates with CRPC development in transurethrally resected PCa specimens and a well-differentiated growth architecture in primary HNPC. In keeping with non-salvage treated tumors having a more aggressive phenotype which warranted early ADT commencement, the data suggested that low ADRB2 was prognostic for poor responses to treatments targeting the androgen receptor signaling axis. The data further indicate that bicalutamide treatment may have masked the prognostic value of ADRB2 for CRPC development. Accordingly, we have shown beforehand that bicalutamide treatment lowers ADRB2 mRNA expression in PCa cell lines. In conclusion, we postulate that β-adrenergic signaling promotes AR activity and luminal differentiation in HNPC. Upon AR blockade, poorly differentiated tumors with low ADRB2 expression more rapidly adapted and recurred, which in turn primed them to resist ADT.

6. Ethical considerations

The use of patient material and mice in this thesis conformed to ethical and responsible research practice. In paper I, the ADT-TMA and use thereof was approved by the Regional Committees for Medical and Health Research Ethics (REK, 2009/1028). In paper II, all patients included on the TMA provided informed consent, and the study was approved by REK (434-04153,S-04153c, 2009/373,09/00450-2/bso), the Oslo University Hospital Research Support Service (2011/3286), and Lund University, Sweden (494/2005). In Paper III, the candidate was granted access to DIPS by the Privacy Ombudsman to extract data from a group of patients deemed eligible for inclusion. The use of cDNA microarray analysis on patient samples to identify markers for ADT response in Paper III was approved by REK (2017/773).

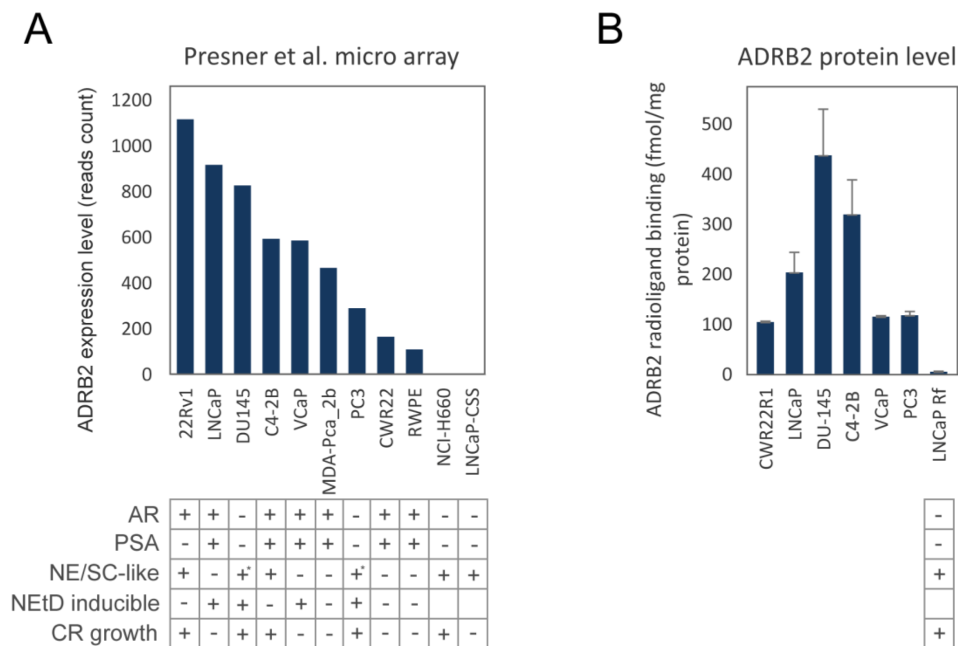
All patient data was stored on secure servers within the Oslo University Hospital servers (2017/773 and ePhorte 2017/8876) or TSD (University of Oslo), and data that could identify individual patients (such as identification numbers) was promptly removed following identification of eligible subjects. Paper code lists were safely stored. Data imported into TSD were de-identified and encrypted before importing using a password-protected USB stick (IronKey).

The use of the non-obese diabetic severe combined immunodeficiency gamma (NSG) xenograft model both in Paper I and II was approved by Mattilsynet (FOTS ID 7132). For these studies, power analyses were performed based on a pilot study to minimize the number of mice needed while including enough subjects to observe statistically significant changes. The choice of using mouse models for both Paper I and II was based on better mimicking the tumor microenvironment. The use of such preclinical model systems can better bridge the gap between laboratory and clinic than what is achieved using cell line monocultures. As the goal of both Paper I and II was to understand mechanisms of therapy resistance to ADT, the use of castration *in vivo* in multiple subjects allows for more heterogeneous responses to the treatment, which more closely resembles what is observed in the clinic. The mice were given daily supervision and biweekly tending/grooming. Day/night cycles were kept at 12h, the temperature was kept at 22 °C, and the mice were fed daily. The mice were kept in environmentally enriched cages (cardboard housing, tubes, running wheel) with a maximum of eight mice per cage. Painful procedures were kept at a minimum. Upon surgical procedures, sacrificing, or when needed for other reasons, pain relief (e.g. analgesics) or gas anesthetics were given to the mice.

7. Methodological considerations

7.1. Model systems

The cell lines used in the experimental work included LNCaP (Paper I, II and III), VCaP (Paper II), PC-3 (Paper II and III), LNCaP C4-2B, DU145 and RWPE-1 cells (Paper III). The primary aim of this thesis was to elucidate mechanisms of resistance towards AR-targeted therapies, and we therefore primarily utilized hormone-sensitive prostate cancer models. This choice is reflected by our continual use of the LNCaP cell line model. LNCaP cells are androgen-responsive and sensitive to androgen depletion (Figure 7.1), and abundantly express AR with a mutation in its LBD [343]. LNCaP cells display a luminal-like gene expression profile [180], express RB1 [344] and wild-type *TP53* [345], and high ADRB2 mRNA and protein levels. LNCaP models NEtD upon multiple drug and treatment regimens, which makes it suitable for studying androgen independence-driven resistance mechanisms and possibly also lineage plasticity in relation to ADRB2. However, as discussed in chapter 7.2, there is controversy regarding whether LNCaP cells truly recapitulate clinical prostate cancer cells undergoing NEtD.



	22Rv1	LNCaP	DU145	C4-2B	VCaP	MDA-Pca_2b	PC3	CWR22	RWPE	NCI-H660	LNCaP-CSS	LNCaP Rf
AR	+	+	-	+	+	+	-	+	+	-	-	-
PSA	-	+	-	+	+	+	-	+	+	-	-	-
NE/SC-like	+	-	+*	+	-	-	+*	-	-	+	+	+
NEtD inducible	-	+	+	-	+	-	+	-	-			
CR growth	+	-	+	+	-	-	+	-	-	+	-	+

Figure 7.1. ***Phenotypic characteristics and ADRB2 expression levels in various prostate cancer cell line models. A****. Gene expression data (RNA-seq) was downloaded from Prensner et al. (GSE25183) [254].* ***B****. ADRB2 radioligand binding in different cell lines is shown as mean of n≥3 biological replicates ± SEM. For each cell line, in-house and published data were used to determine phenotypic characteristics.* **PC-3 and DU145 are occasionally termed model systems for NE/SC-like prostate cancer [346, 347]. N/A indicates that the cells already display NEtD under basal conditions. CR = castrate-resistant, NE/SC-like = neuroendocrine/small-cell-like, LNCaP-CSS = LNCaP cells incubated long-term in charcoal-stripped serum.*

To substantiate results obtained with LNCaP cells, we also utilized VCaP cells, which also are androgen-responsive and androgen-sensitive (Figure 7.1) and display a luminal morphology. VCaP cells have a loss-of-heterozygosity mutation in *TP53* [345]. While treatment with cAMP-elevating agents, ADRB2 overexpression or androgen withdrawal led to NEtD in nearly the whole population of LNCaP cells, we observed that only a fraction of VCaP cells transdifferentiated upon the same treatments. This underscored that while the LNCaP model may represent a suitable model to study NEtD, it only represents a minor fraction of the broad landscape of prostate cancers. To further the scope of our findings, we utilized AR-negative, TP53-null, RB1 positive PC-3 cells [186], sometimes suggested to model NE/SC prostate cancer [346, 347]. Here, multiple cells did not display morphological alterations reminiscent of NED upon androgen depletion. While ADRB2 overexpression led to the majority of cells displaying a neuronal morphology, the cells were not responsive to ADRB agonists which was in accordance with their low ADRB2 protein levels (Figure 7.1). The PC-3 model gave us additional insights into whether ADRB2s role in NEtD was dependent on AR: The fact that ADRB2 overexpression induced neurite outgrowth in AR-negative PC-3 cells showed that we could isolate an ADRB2-specific effect on NEtD independently of the AR.

By ADRB radioligand binding assay and re-analyses of RNA-seq data from multiple prostate cancer cell lines, we found that ADRB2 levels varied substantially. With some exceptions, cell line models with more aggressive behavior, androgen depletion-resistance and NE features displayed lower ADRB2 levels than their counterparts. Encouragingly, the latter finding was supported by the progressive downregulation of ADRB2 mRNA levels in increasingly treatment-resistant clinical tumors.

Both androgen-sensitive and -insensitive clones have been identified in LNCaP monoculture [348], but this heterogeneity is minuscule compared to the complex heterogeneity of patient tumors with their associated TMEs. To better mirror clinical tumor behavior, we subcutaneously injected high- and low-ADRB2 LNCaP cells in NSG mice. Here, we observed castrate-resistant growth after a brief lag period following castration, which is not observed in 2D LNCaP monoculture. Thus, our xenograft model recapitulated the clinical augmentation of CRPC better than *in vitro* monoculturing. Multiple factors, such as vascularization and innervation, are dependent on the grafting site [349], and the subcutaneous TME recapitulates clinical tumors to a lesser extent than e.g. orthotopic grafting. Thus, our choice of model system has multiple weaknesses. Ultimately, it would have been of interest to utilize PDX models in which matched tissue sections could be interrogated for their ADRB2 before engraftment. Several PDX models have been shown to recapitulate the heterogeneity and therapy responsiveness observed in their respective patient donors [349]. With these models in hand, we

could establish whether hormone-naïve ADRB2 levels associated with differential responses to castration.

7.1.1. Stable transfection with shRNAs targeting ADRB2

Proper selection of the insert sequences is critical for achieving gene-specific knockdown and preventing non-specific binding [350]. By using multiple shRNAs containing different insert sequences, it is highly unlikely that off-target effects will be present in all the stably transfected cell lines. The knockdown efficacy of the different shRNAs targeting ADRB2 in LNCaP cells varied from 50-95% at the mRNA level and 50-85% by radioligand receptor binding assay. These differences may be due to multiple factors: First, the different insert sequences may have different affinity for the ADRB2 mRNA. Low binding affinity could theoretically lead to less frequent Dicer binding, and as the sequences are generated *in silico*, they may target less sterically available areas of the mRNA. Second, the efficacy of the plasmid delivery may have been different between the cell lines by chance. Third, potential toxic effects of highly efficient ADRB2 knockdown may have led to the selection of clones exhibiting less efficacious knockdown. We attempted to generate stable ADRB2 knockdown cells in PC-3 and DU145, and although we could generate viable antibiotic-resistant clones in both cell lines, neither showed ADRB2 knockdown at the mRNA level. Whether these findings relate to selection due to detrimental effects resulting from lowered ADRB2 expression levels, or if compensatory mechanisms masked ADRB2 downregulation, remains unclear.

7.2. Assessment of neuroendocrine transdifferentiation

Neurite quantification is a common method to assess the extent of neurite outgrowth in cell line model systems. While the process of neuritogenesis is not confined to NEtD, it often appears concomitantly with an elevation of NE-markers both *in vitro* and in prostate cancer tumors.

Neurite outgrowth was measured semi-manually (paper II) using the NeuronGrowth plug-in [351] in ImageJ. Briefly, cell culture images were captured using the IncuCyte FLR software or a fluorescence microscope. High-resolution images were obtained at selected time points, and neurite quantification was performed using a neurite tracer tool as shown in Figure 7.2. Here, neurites were tracked by user-defined start- and endpoints, yielding the length of each neurite. The number of cells from each image field was then counted manually, and the total length of neurites was field-wise related to the number of cells.

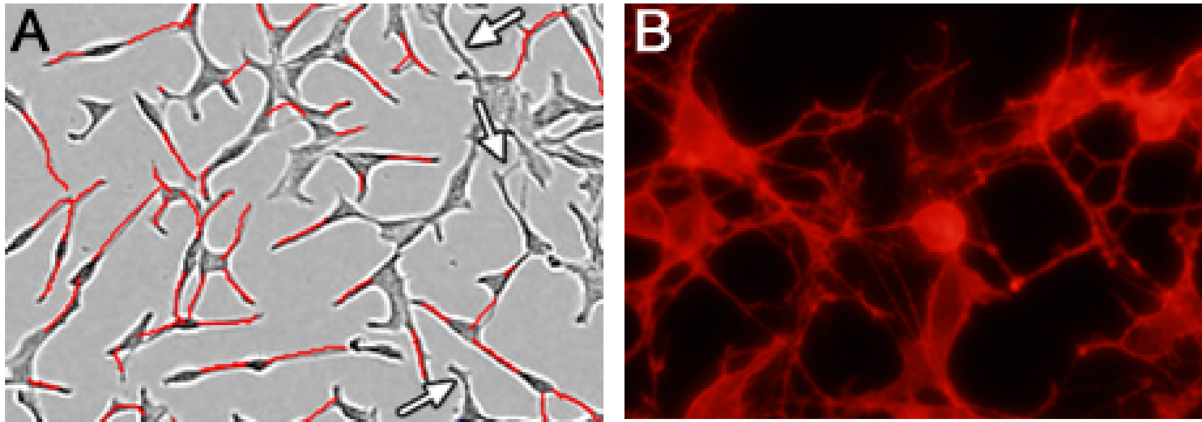


Figure 7.2. ***Neurite outgrowth in vitro. A****. A small field of a light microscopy (phase-contrast) image LNCaP shCtrl cells incubated in 2% CSS for two days captured using the IncuCyte FLR software. The image was opened in ImageJ with the NeuronGrowth plug-in. The red lines indicate the neurites measured manually. Some neurites were not measured at the time of image capturing for clarity (white arrows).* ***B****. ADRB2-FLAG-overexpressing LNCaP cells visualized using an anti-FLAG antibody. Note the extensive neurite protrusions and branching.*

A clear limitation of the procedure used in this work relates to the manual nature of the neurite tracings and cell counting. As can be seen from Figure 7.2, the cell protrusions differed in width (white arrows), and no predefined cut-off for width was set, which may have led to incorrect labeling. Manual counting is also a point of concern, as LNCaP cells tend to grow in aggregates. To amend to these limitations, we performed the experiments in technical and biological triplicates from large fields, plated cells at similar confluences, and selected fields of representative confluence. Differences in cell numbers between the LNCaP shADRB2 cells were not systematically different from shCtrl cells (two-sided t-test all $P > 0.05$ for all treatment conditions). Encouragingly, the results could be reproduced using a more automated procedure in an independent laboratory (University of Eastern Finland). Phase-contrast images of shCtrl and shADRB2 incubated in CSS were captured, and neurite lengths and branch points were quantified using the NeuroTrack Software module for IncuCyte S3. The findings were complemented by analyses revealing a reduced number of branching points in shADRB2 cells.

The extent of neurite outgrowth was correlated with expression of the NE-markers NSE and Tubulin beta-3 class III (TUBB3) in shCtrl and shADRB2 cells. ADRB2 overexpression in LNCaP cells, on the other hand, induced a marked neurite outgrowth but did not lead to elevated NE-marker expression. We hypothesized that this could be due to a "ceiling effect", in which NE-marker levels were at their peak levels to begin with. However, ADRB2 overexpression in shADRB2 cells, PC-3 cells and VCaP cells did not affect NE-marker mRNA or protein expression. These findings were surprising, as we have observed neurite outgrowth and elevated NSE expression upon isoproterenol exposure in LNCaP

cells. The ADRB2-overexpressing cells retained their extensive neuronal morphology (Figure 7.2 B and Figure 3.7 F), but attempts to generate stable ADRB2-overexpressing cells failed as the cells were non-proliferative and readily detached from the culture plate. This could indicate that the constitutive activity of the abundantly expressed ADRB2s (verified by immunofluorescence) was toxic to the cells, which prevented transcription of NE-markers. To test this hypothesis we attempted to transfect with a lower fraction of the ADRB2 overexpression plasmid. We observed that even with the lowest ADRB2-dosage sufficient to induce morphological changes, the cells stopped proliferating and NE-markers remained unaffected. A consensus characterization of NE-transdifferentiated cells is lacking, and the NE prostate cancer subtypes may express different sets of NE-markers. Although we did not observe elevated SOX2, CHGA or SYP levels by qRT-PCR or immunofluorescence, we cannot rule out that other NE-markers were elevated in response to ADRB2 overexpression.

While we could establish a functional role of ADRB2 in NEtD and a consistent preclinical and clinical downregulation of ADRB2 expression in t-NEPCs (Paper II), our experiments could not prove a causal, sequential relationship between NEtD and t-NEPC. Several studies indicate that a more durable and multimodal treatment challenge is necessary to more closely recapitulate true proliferative t-NEPC development. To this end, proliferative LNCaP-derived NE subclones such as NE1.3 have been generated by picking single cells from low-passage LNCaP cells that had been maintained under androgen depleted conditions for 4-6 months [352]. The NE1.3 cell line displays castrate-resistant growth, ADRB2 downregulation and elevated expression of CHGA, CHGB and NSE [196, 330]. It is not known however how well this model recapitulates clinical t-NEPC emergence, despite the genomic and phenotypic resemblances. Although we used publicly available gene expression datasets to identify the role of ADRB2 in t-NEPC emergence (chapter 7.3), we would probably have benefitted from utilizing models that more closely recapitulate true t-NEPC emergence.

7.3. *In silico* analyses of publicly available clinical gene expression datasets

A large number of preclinical and clinical studies published append genomic and/or transcriptomic data deposits, including RNA-seq and microarray data. These data sets may be deposited on databases such as Gene Omnibus (GEO) [353] (both preclinical and clinical data) and cBioPortal (only clinical, www.cBioPortal.org [354, 355]). Clinical datasets may be accompanied by histological parameters, and often also baseline and follow-up clinical and pathological parameters.

There are multiple benefits of analyzing publicly available datasets: Primarily, it serves as a means to validate experimental findings while giving further biological insights and drive hypothesis generation. When coupled with baseline characteristics or clinical follow-up data, such repositories can help bridge the gap between laboratory and clinic without having to initiate costly studies.

There are several caveats and limitations to these analyses, however: First, transcriptomic analyses are performed on a plethora of platforms, and there is technical variability between experiments formed on the same platform. This warrants careful considerations of batch effects should one want to merge different datasets. For this reason, the datasets queried in this work were evaluated independently. A second challenge relates to data normalization: Whereas some datasets contain already normalized data, others are only available as raw data. The latter group must be normalized. In this work, non-normalized microarray datasets were normalized using either Robust Multi-Array Average (RMA) or MAS5 in the *affy* package [356] or SCAN-UPC in Bioconductor [357] in R (v3.2.4-3.5.0). These methods give background correction and quantile normalization, but were found to sometimes yield slightly different outputs. Third, the selection and exclusion criteria for clinical datasets are not always explicitly stated. In these cases, the dataset may or may not be randomized, and thus it will not be known whether it embodies a representative patient population. This may explain the large discrepancies in reported frequencies of metastases with t-NEPC, for instance. A fourth and more general limitation of using transcriptomic data is that gene expression levels do not necessarily reflect protein levels which more closely reflects the cell phenotype, but rather the transcriptomic regulatory landscape. In Paper II, we found that ADRB2 immunohistochemical staining in a tissue microarray (TMA) was inversely correlated with Gleason grade, and that high ADRB2 levels were associated with more rapid BCR in patients with primary Gleason grade 4. When we queried the TCGA provisional [291] and MSKCC 2010 [187]) clinical transcriptome datasets, ADRB2 mRNA level was associated with neither Gleason grade nor BCR. Despite this, we cannot rule out that the ADRB2 immunohistochemical staining intensities in these datasets may show the same associations that we found in our tissue TMA. To date, we have not investigated to which degree ADRB2 mRNA and protein levels correlate.

As a general note, tissue confounding may lead to biased conclusions. The samples used for RNA extraction may not exclusively contain tumor tissue but may contain benign cell populations as well as adjacent stroma. Although some studies control for this, it is not reported in other studies, which makes integration of results from multiple datasets a daunting task.

Primary prostate cancers, as were assessed in our transcriptomic profiling study (Paper III), display multifocality and interfocal genomic and transcriptomic heterogeneity which may be confounded by adjacent stromal or benign compartments [358, 359]. Although we used pathology-guided macrodissection of areas containing tumor with the final assigned Gleason score, the selected foci may not necessarily represent the cell population that recurred following RP or that seeded to form a metastasis prior to surgical removal of the prostates [38]. Hence, the transcriptomic profiles may vary between intra-tumoral foci, which limits the potential to generalize the findings. We are

currently working on partly addressing this issue by generating a TMA containing several spots from distinct foci from each patient and verify selected markers by immunohistochemistry.

7.4. The Curse of Dimensionality

DNA microarray technology allows for the simultaneous measurement of relative expression levels of a large number of genes in a biological sample. In this section, I will use gene expression profiling by array as an underlying theme and introduce relevant statistical considerations important for the discovery of transcriptomic features associated with CRPC emergence (Paper III).

There are two main ways to learn from the data in the setting of machine learning, namely unsupervised and supervised learning. In unsupervised learning, one either has no, or assumes no outcome variable. Thus, unsupervised learning aims to describe the input measures [360]. In supervised learning, on the other hand, one uses the input data to predict an outcome measure. For our retrospective study, we have collected variables such as CRPC development in our training set, which allows us to learn from the input's (an expression matrix of 12,979 unique genes x 37 patients) association with either a categorical (e.g. binary) or time-dependent variable in a supervised manner.

When performing machine learning of a dataset with nearly 13,000 input metrics, we are analyzing at 13,000 dimensions. This infers that when adding additional dimensions (e.g. we include x more probe sets), we will need $N = x^2$ more observations (here tumor samples) to make a meaningful model. This phenomenon is termed the *curse of dimensionality*, and occurs when $x \gg N$. In our study, we generally aimed to identify transcripts which' abundances are differential between patients with short and long PFS, albeit with a small N. After constructing a statistical test for each gene x_j, we can *by chance* expect a large number of genes to show a low *p*-value. In fact, if we assume all the measured genes to be independent of the outcome measure, one would here expect 649 genes to have a *P*-value < 0.05 at an α-level of 0.05. Hence, a mean of correcting for multiple testing should be applied to reduce the number of false positives: The Benjamini & Hochberg False Discovery Rate (FDR) yields the expected proportion of false positives. By setting an FDR threshold, one can then identify genes with a higher chance of being true positives.

A large caveat with adjusting for multiple testing is the possibility of having false negatives. When training a prediction model, adjusting the FDR threshold upwards may be beneficial to reduce this caveat, however, especially when the study is explorative [360]. In this study, no genes were significantly associated with primary or secondary endpoints after FDR-adjustment. Although the *p*-value distributions generally showed only a small trend towards enrichment of low values, the explorative nature of the study led us to be less stringent. For example, by using approaches such as

Gene Set Enrichment Analysis (GSEA) and calculation of gene signature scores, the sum of trends for a gene set or signature can give biological insights. With our *a priori* hypothesis that ADRB2 would associate with the effect of drugs targeting the AR signaling axis, we could also here be less stringent in terms of correcting for multiple testing.

As expression levels of many genes are likely dependent on clinical and pathological variables, we performed multivariable Cox proportional hazards regression to adjust for the markers independent prognostic value. To have a clinical utility, a biomarker must add prognostic value on top of established risk parameters. As there is currently limited information about whether tumor-related characteristics associate with resistance to androgen-targeted therapies, we chose to adjust for risk factors for e.g. biochemical recurrence. Importantly, due to the strong association observed between salvage bicalutamide use and expedited CRPC, and the fact that bicalutamide was administered before the follow-up time started in these analyses (i.e. onset of ADT), this treatment was also included in multivariable models. Generally, the utility of such models may be limited by the eligibility criteria for inclusion into the study and the limited sample size. Hence, within the material analyzed, univariable models may independently yield valuable information, and were therefore reported.

It should be noted that gene expression profiles found to predict outcome in one study commonly fail to do so when tested in independent datasets. This is a major limitation of paper III, where $x \gg N$ and generalization of the prediction model may be considered low. The prevalence of radically operated patients who received ADT within the screened population was low, however. Hence, when considered independently, the results should primarily be interpreted as exploratory and hypothesis-generating.

Ultimately, by testing our trained data (e.g. ADRB2 or a predictive model) in independent cohorts the validity of the findings can be assessed. To identify predictive biomarkers for clinical utilization a prospective study should be utilized in which patients are directed towards different treatment strategies based on the predictive model. If verified in independent cohorts, our present study may lay the grounds for such studies.

8. Discussion

The overall aim of this thesis was to elucidate molecular mechanisms of resistance to androgen targeted therapies in advanced prostate cancer, with emphasis on the role of the β_2-adrenergic receptor. In brief, we show that pre-treatment ADRB2 levels associate with differential outcomes upon androgen targeted therapy challenge in preclinical model systems and patient tumors. We find that the effect of ADRB2 level and its signaling pathway is dependent on AR-status and prior treatments targeting the AR signaling axis.

8.1. ADRB2 in development of CRPC adenocarcinoma

A central finding in this thesis was that prostate cancers with low ADRB2 expression and/or protein levels were associated with resistance towards androgen-targeted therapies. Specifically, we show that low ADRB2 immunohistochemical staining in transurethrally resected tumor tissue specimens associated with rapid CRPC. None of the patients included in the study showed clinical or biochemical characteristics implicative of t-NEPC, and all patients had rising PSA upon CRPC emergence. In keeping with this, as well as the fact that the patients' follow-up period was in the pre-abiraterone/enzalutamide era, this cohort most probably exclusively contained patients developing CRPC adenocarcinomas. We further show that primary, hormone-naïve tumors with low ADRB2 mRNA levels more rapidly recurred on salvage bicalutamide. Using preclinical model systems, we showed that prostate cancer cells expressing low ADRB2 levels better retained residual or endogenously produced androgen. When challenged with androgen depletion, low-ADRB2 cells were more androgen-responsive towards reintroduced androgens and more rapidly recurred as CRPC when grown in a xenograft model undergoing castration. We discovered that ADRB2 depletion led to downregulation of the UDP-glucuronosyltransferase 2B (UGT2B) family of genes, including UGT2B15 and UGT2B17 which are well documented to glucuronidate and inactivate androgens [97, 98]. These findings suggested that UGT2B downregulation promoted reactivation of androgen signaling under pressure from androgen-targeted therapies.

We showed that β-adrenergic stimulation promoted AR activity in AR-positive cells, a finding which supported previous studies linking the β-adrenergic signaling pathway to AR transactivation ([137] and references therein). A similar relationship is likely to present in primary, hormone-naïve tumors, as high ADRB2 mRNA levels associated with a well-differentiated growth pattern and enrichment of androgen-responsive genes (Papers II and II). These findings implied that high-ADRB2 hormone-naïve prostate cancer cells should have higher AR signaling. This is seemingly in conflict with low-ADRB2 cells being more primed to resist androgen depletion. However, our findings illustrated that

depletion of ADRB2 conferred adaptive mechanisms that compensated for a lack of β-adrenergic signaling-mediated AR transactivation. In fact, ADRB2 depletion made the cells more responsive towards androgen re-supplementation and more resistant to ARPIs than high-ADRB2 cells (Papers II and III). Thus, it is plausible that ADRB2 downregulation leads to a rewiring of the cells towards better resisting androgen depletion and AR pathway inhibition. A relationship between ADRB2 and androgen elimination was also evident in patient tumors, as ADRB2 and UGT2B15 and UGT2B17 protein levels positively correlated in patient tumors from two independent cohorts. Furthermore, weak UGT2B15 staining was, like ADRB2, associated with expedited CRPC development. Hence, the inverse association between ADRB2 levels and CRPC emergence may relate to lowered glucuronidation activity in low-ADRB2 expressing tumors.

We could not determine how ADRB2 depletion caused the substantial downregulation of numerous genes within the UGT2B family. Both UGT2B15 and UGT2B17 have been reported to be negatively regulated by active AR signaling [99, 100], and the UGT2B genes are highly homogenous [104]. Hence, the downregulation was probably not reduced due to ADRB2 depletion-mediated loss of AR-activity, but possibly via a yet to be identified factor balancing the sympathetic and androgenic input in prostate cancer cells, as discussed later. There is to date no consensus on whether the UGT2B family of genes promotes prostate cancer progression and/or CRPC, or whether their enzymatic activity towards non-steroidal molecules is of more importance [361]. Our data and results from a recent study [362] do however point towards that UGT2B loss or downregulation confers resistance during androgen targeted therapy through enabling better preservation of up-taken or *de novo* synthesized androgen in a low-androgen milieu.

Aside from the mechanism involving downregulation of the UGT2B family of genes, we have unpublished data showing that ADRB2 depletion may alter other metabolic pathways. As β-adrenergic signaling regulates steroidogenesis in multiple model systems and tissues [268-271, 363], we sought to investigate whether this pathway could be altered upon stable knockdown of ADRB2 in prostate cancer cells. We observed that ADRB2 knockdown cells expressed higher levels of genes involved in the steroidogenic pathway, including ATP citrate lyase (ACLY) and fatty acid synthase (FASN) (data not shown). Low-ADRB2 LNCaP cells xenografted into mice had significantly higher cholesterol levels than high-ADRB2 cells after castration, indicating a functional effect of gene upregulation of key lipid biosynthesis/steroidogenesis enzymes. Corroborative of this, low-ADRB2 cells challenged with androgen depletion also displayed higher cholesterol levels. As increased steroidogenesis is a widely perceived mechanism of sustaining androgen levels to resist ADT, these results supported that loss of ADRB2 induces compensatory mechanisms to sustain androgen signaling and favors reactivation of the AR signaling axis under androgen deprivation therapy. As the

majority of CRPCs are characterized by reactivation of AR signaling, our low-ADRB2 model system represents a model of CRPC adenocarcinoma.

8.2. ADRB2 and luminal differentiation

An explanation for why the mentioned compensatory mechanisms occur may lie within the bidirectional dependencies of the β-adrenergic and AR signaling axes, and the remarkable reliance of prostate cancer cells on androgens. AR- and β-adrenergic signaling target genes show considerable overlap [326], and AR activation has been suggested to be heavily reliant on active Gαs-cAMP-PKA signaling [156]. Furthermore, testosterone leads to increased ADRB2 protein levels in rat prostates [299], and the AR has been shown to non-genomically activate the cAMP-PKA signaling cascade in pancreatic β-cells [364]. Intriguingly, sympathetic nerves producing catecholamines acting on ADRB2s are, like androgen stimulation, critical for prostate cancer oncogenesis and progression [308, 317]. As mentioned, ADRB2 mRNA levels positively correlate with expression of androgen-responsive genes and luminal differentiation genes both in primary hormone-naïve tumors, and CRPCs (Papers II and III). β-adrenergic signaling may thus promote AR activity and transcription of canonical genes within the full-length AR cistrome in hormone-naïve tumors. Hence, it seems plausible that cells committed to luminal lineages may counteract the inhibition of the androgen signaling axis by activating the β-adrenergic signaling pathway.

Prostate cancer progression and therapy resistance associates with loss of differentiation and luminal lineage commitment. The dependency of luminal differentiated prostate cancers to β-adrenergic signaling is further underscored by ADRB2's consistent downregulation following androgen depletion: When xenografted into NSG mice, both high- and low-ADRB2 expressing tumors had undetectable ADRB2 protein levels following castration, consistent with our previous data [255] and multiple studies with appended gene expression profiling data showing that castration reduces ADRB2 levels [187, 365-367]. We showed that low-ADRB2 tumors displayed longer tumor latencies and reduced growth compared to high-ADRB2 cells in intact NSG mice. This may relate to high-ADRB2 cells being more differentiated and thereby more primed to colonize the tumor microenvironment. Another explanation for the delayed time to tumor take may be that high-ADRB2 cells were more responsive to sympathetic stimulation, which is has been documented to be essential for prostate cancer development [308, 317].

8.3. ADRB2s involvement in NEtD and t-NEPC emergence

Activation of the β-adrenergic receptor and its' downstream signaling cascade is well established to prompt neuroendocrine transdifferentiation of prostate cancer cells [213, 214]. Accordingly, we

showed that ADRB2 downregulation in LNCaP cells reduced β-agonist-induced NEtD and expression of NE markers in hormone-naïve cells. While high-ADRB2 LNCaP cells readily developed NEtD following androgen depletion, low-ADRB2 cells displayed abrogated NEtD dose-dependently of pre-treatment ADRB2 levels. Similarly, in the NSG xenograft model, high-ADRB2 tumors developed NED to a higher extent than low-ADRB2 tumors after castration. Nouri and colleagues have suggested that NEtD occurs via an intermediate stem-like reprogramming [368]. In keeping with this, our findings indicate that the ADRB2 level in hormone-naïve prostate cancer cells was determinant of lineage fate following androgen depletion. While this finding supported that low-ADRB2 cells were committed to an adenocarcinoma-CRPC, NE-negative lineage as indicated in our TUR-P tissue microarray, it also revealed that high-ADRB2 cells were more prone to develop NED. By tracking individual cells we found that neuroendocrine transdifferentiation rather than clonal selection occurred. Importantly, while high-ADRB2 expressing xenograft tumors displayed more NE-marker staining after castration, both high- and low-ADRB2 expressing tumors showed a near-complete loss of ADRB2 staining. This corroborated the notion that pre-castration rather than post-castration ADRB2 levels were determinant of lineage fate.

High-ADRB2 tumors in our xenograft model tended to have more Platelet and endothelial cell adhesion molecule 1 (CD31)-positive cells than low-ADRB2 tumors after castration. This falls in line with studies showing that neuroendocrine tumors display increased vasculature [369]. Furthermore, two studies showed that β-adrenergic stimulation led to increased CD31 staining and drove angiogenesis in prostate cancer mouse models [196, 318]. Thus, the high-ADRB2 expressing tumors may have promoted emergence of NE-features, which involves increased tumor vascularization.

Although we do not establish the underlying mechanism explaining why low-ADRB2 cells failed to undergo androgen depletion-induced NEtD, their high androgen responsiveness may have maintained the cells' luminal differentiation despite lowered cAMP-PKA-mediated AR transactivation. A more direct role of ADRB2 in driving NEtD is also plausible: ADRB2 overexpression induced extensive neurite outgrowth in both AR-positive and AR-negative prostate cancer cell lines. Interestingly, ADRB2-overexpressing cells did not display elevation of NE-markers. NEtD in prostate cancer cell lines is widely characterized by induction of neurite outgrowth and elevation of NE markers [211, 370, 371]. Whether these processes are independent, dependent, or sequential, is not known to date. Our experiments indicate that ADRB2 overexpression may be biased towards driving neurite outgrowth via incrementing cAMP-PKA-RhoA-ROCK signaling [325] rather than promoting expression of NE-markers. The ability of ADRB2 overexpression to promote neurite outgrowth in PC-3 cells, thus independently of AR signaling, supported a direct neurite outgrowth-promoting role of ADRB2. Furthermore, forskolin induced neurite outgrowth in both high- and low-ADRB2 cells, which

suggested that ADRB2 promoted neurite outgrowth in a cAMP-dependent manner. Interestingly, whereas depletion of ADRB2 led to a notable reduction of NEtD following androgen depletion, NE-features were also less apparent in low-ADRB2 cells in presence of androgen. Collectively these findings indicated dual effects of β_2-adrenergic signaling on NEtD: While active ADRB2-signaling may drive neurite outgrowth via the RhoA-ROCK signaling cascade, lowered ADRB2 signaling reduces both neurite outgrowth and NE-marker expression, particularly under challenge with androgen depletion.

In our search for the underlying mechanisms explaining how ADRB2 depletion reduced NEtD, we observed that low-ADRB2 cells had elevated activity of the canonical Wnt/β-catenin signaling pathway. Increased β-catenin stabilization by GSK3β inhibition abrogated NEtD and reduced expression of NE-markers and neuron differentiation genes, which was concordant with another study showing that knockdown of GSK3β resulted in abrogated neurite outgrowth in NGF-treated PC12 cells [372]. This suggested that elevated Wnt/β-catenin could contribute to abrogated NEtD. The Wnt pathway has been shown to be overactive in enzalutamide-resistant prostate cancer cell lines, and activation of Wnt/β-catenin was recently reported to promote enzalutamide resistance [373]. The non-canonical Wnt11 protein, which activates the RhoA-ROCK pathway, decreases beta-catenin stabilization [374] and inhibits the canonical Wnt pathway [375]. In LNCaP cells, Wnt11 promoted NEtD in a PKA-dependent manner [375], supporting the notion that canonical Wnt signaling may abrogate NEtD. The role of Wnt signaling is likely context-dependent [376], which may explain the conflicting results regarding its role in NEtD [377, 378]. We observed in our study that the canonical Wnt pathway was increased upon androgen depletion, in concordance with another study [378], suggesting that this signaling pathway may both inhibit NEtD in hormone-naïve cells while promoting sustained NEtD in androgen depletion-challenged cells.

Our differential gene expression analysis of high- and low-ADRB2 LNCaP cells revealed downregulation of genes annotated *neuron differentiation* in ADRB2-depleted cells. Among these, SLIT And NTRK like family member 3 (SLITRK3), CD24 molecule (CD24) and Jagged canonical Notch ligand 1 (JAG1) have been shown to be upregulated following androgen depletion of LNCaP cells [220]. SLITRK3 has been shown to be critical for presynaptic differentiation [379], and JAG1 expression associated with poor responses to androgen withdrawal in a preclinical model [348]. Furthermore, Kelch like family member 1 (KLHL1), also downregulated in low-ADRB2 cells, was reported to be positively regulated by GSK3β and to participate in formation of neurite outgrowths [372]. This suggests that these genes may have a function in the maintenance of NEtD, and that their downregulation may inhibit NEtD from occurring.

The array of studies showing that inhibition of the AR signaling axis promotes NEtD, and the frequent AR downregulation/negativity in t-NEPCs, suggest that the loss of AR signaling is a prerequisite for NEtD. In keeping with the bidirectional dependencies of β-adrenergic and AR signaling, our data suggests that also active β-adrenergic signaling is essential for NEtD. It could be contemplated that androgen depletion causes a transient induction in ADRB signaling as a means to compensate for loss of AR activity. Accordingly, we have unpublished data showing that androgen depletion and β-agonist exposure of LNCaP cells induce a durable cAMP elevation, which is supported by another study [380]. ADT is associated with hot flashes [381], and plasma concentrations of norepinephrine and its metabolites are elevated before and during hot flashes [382, 383]. However, while the β-blocker propranolol inhibited β-agonist-induced NEtD, it did not abrogate androgen depletion-induced NEtD in high-ADRB2 cells. Whether the cAMP elevation occurring following androgen depletion takes place in human subjects, how persistent it is, and if it is mediated via β-adrenergic receptors remain to be investigated.

8.4. Mechanism of ADRB2 downregulation

Both β-adrenergic activation and androgen depletion reduce ADRB2 transcript levels. Prolonged agonist exposure to ADRB2s leads to a well-documented desensitization and receptor internalization [274-276]. In this thesis, NEtD and subsequent t-NEPC emergence were shown to associate with diminishingly low ADRB2 transcript levels in preclinical models and patient material. T-NEPC emergence is associated with total loss of luminal differentiation exemplified by AR-indifference and resemblance to small-cell cancers and was the disease subtype showing the lowest ADRB2 expression levels. Moreover, a recent meta-analysis on top differentially expressed genes in t-NEPC identified ADRB2 as among the top downregulated genes [225]. The progressive downregulation of ADRB2 following disease progression and its' inverse association with Gleason grade reckoned that ADRB2 could merely be a differentiation marker. In the study by Nouri *et al.,* switching to a N/NC stem-transition medium led to a suppression of androgen signaling, loss of differentiation and enzalutamide resistance in several prostate cancer cell lines [368]. This intermediate state was suggested to be critical for transdifferentiation to neural lineages, and reprogramming of all four cell line models led to significant ADRB2 downregulation. Correspondingly, therapy-resistant prostate cancers increasingly lose differentiation, and ADRB2 is progressively silenced by H3K27me3 in a model for neuron differentiation [18]. Taken together, these data suggest that ADRB2 downregulation may be a prerequisite for transdifferentiation to occur and to maintain NE lineages, rather than only being a marker of differentiation, which indeed was what we observed using cell lines and xenograft models.

Further evidence that ADRB2 plays a functional role in NEtD rather than being a marker of differentiation relates to its close association with EZH2. EZH2 silences ADRB2 through its PRC2-dependent H3K27 trimethylation activity [195, 349, 384], is commonly upregulated in NE cells and t-NEPC tumors [33, 196] and is transcriptionally repressed by RB1 [193]. The relationship between EZH2 and ADRB2 may not be unidirectional, however, but rather reciprocal: The ADT-driven increase in EZH2 activity was recently shown to be dependent on PKA-CREB signaling [196]. In the same study, isoproterenol treatment increased H3K27me3 levels in multiple cell lines while propranolol inhibited the enzalutamide-driven increase in H3K27me3. Although we and others have observed that isoproterenol may favor ADRB2 desensitization and internalization, this represents another mechanism of ADRB2 downregulation. Importantly, this study suggests that activation of β-adrenergic receptors may be one of the early events in NEtD and t-NEPC emergence. We have however observed that the β-blocker propranolol only inhibited β-agonist-induced, but not androgen depletion-induced neurite outgrowth, which may conflict with this notion. A possible model explaining these conflicting findings is that androgen depletion reduces AR activity while increasing cAMP, which may induce neurite outgrowth. The fact that propranolol did not decrease neurite outgrowth suggests that androgen depletion-mediated cAMP elevation is not ADRB2-mediated, but rather driven by activation of other signaling pathways or dysregulation of factors mediating cAMP levels. For example, one study reported that the cAMP phosphodiesterase 4D7 (PDE4D7) is downregulated in androgen-independent prostate cancer [152], and may therefore represent a possible mechanism of maintained cAMP signaling in this prostate cancer subtype.

We have shown that ADRB2 regulation is highly sensitive to treatments limiting AR activity. By re-analyzing the RNA-seq data appended to Labrecque *et al.* [171] (data not shown), we observed that ADRB2 downregulation in metastatic CRPC biopsies was more closely attributed to lowered or lost AR expression rather than acquisition of NE/SC features, as assessed by immunohistochemistry. More specifically, ADRB2 was particularly downregulated in AR^- as compared to AR^+ tumors, but was similar between AR^-/NE^- and AR^-/NE^+ tumors. Furthermore, low AR protein levels were mirrored by a negative enrichment of the Hallmark androgen response gene set. Similarly, in the Aggarwal *et al.* dataset [161], ADRB2 expression was downregulated in NE/SC biopsies but was particularly low in NE/SC biopsies from patients with low serum PSA taken at the time of biopsy (data not shown). The potential utility of supraphysiological testosterone therapy for enzalutamide-resistant prostate cancer [249] suggests that maintaining active AR signaling can inhibit tumor progression upon potent AR-blockade. Whereas the expression of canonical AR target genes such as KLK3 and NKX3-1 are readily increased by androgen supplementation in androgen-deprived cells, as may occur during supraphysiological testosterone treatment, we observed that it did not rescue ADRB2 transcript

levels. Clinical augmentation of CRPC adenocarcinoma leads to reactivation of AR signaling, and ADRB2 was downregulated at this disease stage. The maintenance of ADRB2 downregulation at this disease stage suggests that loss of AR signaling invokes epigenetic silencing of ADRB2. As mentioned, this may be driven by an early event leading to ADRB2 activation which drives EZH2s activity towards depositing repressive marks on the ADRB2 promoter.

Finally, loss *RB1* and *TP53* associates with t-NEPC emergence [33, 186]. Agents conferring elevated cAMP led to inactivation of RB1 via dephosphorylation and downregulation [385, 386] and decreased p53 stabilization by ubiquitination upon ionizing radiation-induced stress [387]. Moreover, β-agonist driven Gαs activation and βarr recruitment and activation has been shown to suppress p53 levels in an AKT-dependent manner [279]. The RB1 and p53 proteins, which putatively bind to the ADRB2 promoter, may exert part of their tumor-suppressive functions through driving transcription of ADRB2 [193, 388]. These findings corroborate the notion that activation of the β_2-adrenergic signaling cascade may be an early event driving lineage plasticity and t-NEPC emergence.

8.5. The β_2-adrenergic receptor in t-NEPC

An alternative mechanism of ADRB2 downregulation may relate to long-term β-adrenergic agonist-induced desensitization of ADRB2. In a hormone-naïve setting, dampened cAMP-PKA signaling should theoretically decrease AR activity, which is a feature of NEtD and t-NEPC. This is supported by the putative activity of the cAMP-PKA-CREB signaling axis following androgen depletion, which intuitively should lead to lowered ADRB2 protein levels. Interestingly, despite the evidently lowered ADRB2 expression in preclinical and clinical tumors with NED/t-NEPC, a growth-inhibitory effect of propranolol has been reported in NEPC xenografts [196]. Taken together with data suggesting that the cAMP-PKA-CREB signaling axis remains active following androgen depletion and emergence of NE/NEPC features [196, 330, 380], it is plausible that β-adrenergic signaling is active and may sustain growth in AR-negative/indifferent prostate cancer subtypes despite downregulated ADRB2 expression. While ADRB1 is downregulated and ADRB3 unchanged in t-NEPCs compared to CRPC adenocarcinomas in the Beltran *et al.* dataset [33], and ADRB2 is the predominant ADRB subtype in prostate cancer cells, propranolol's growth-inhibitory effect was presumably ADRB2-specific.

The possibility that ADRB2 may be expressed at the protein level in t-NEPCs, and the fact that ADRB2 is transcriptionally downregulated also in CRPC adenocarcinomas, implicates that ADRB2 may be expressed at the protein level also in this latter subtype. In our TUR-P cohort, a subset of patients excluded from the primary endpoint analysis had developed CRPC prior to the treatment. ADRB2 staining intensities were not different between CRPCs and non-CRPC tissue specimens (data not shown). Furthermore, ADRB2 staining intensities were also similar among hormone-naïve and LHRH-

treated TUR-P specimens. These findings suggest that prostate cancer cells may retain ADRB2 protein levels despite transcriptional silencing of ADRB2 upon therapy resistance development.

8.6. On the prognostic value of ADRB2

In other solid tumor cancers, ADRB2 levels associate with both favorable and unfavorable outcomes [332-337]. In prostate cancer, Yu and colleagues reported that low ADRB2 immunohistochemical staining in primary tumors associated with clinical failure independently of clinicopathology [195]. This finding was corroborated by our findings showing that low ADRB2 staining intensities and mRNA levels associated with rapid biochemical recurrence after radical prostatectomy and BCR on bicalutamide, respectively. Yu further showed that ADRB2 knockdown transformed benign, AR-negative RWPE-1 cells to an invasive, mesenchymal phenotype. Low ADRB2 tumors may, therefore, have more mesenchymal properties which may promote early regional or distant metastatic spread and therapeutic resistance [389]. This can, in turn, explain why low-ADRB2 tumors more rapidly recur post RP and bicalutamide.

Meanwhile, we have unpublished data showing that activation of β-adrenergic receptors induces migration and NEtD of AR-positive LNCaP cells. These divergent findings suggest that ADRB2s role in prostate cancer is contextual and dependent on AR activity. Specifically, while EMT and NED are characteristics of prostate cancer cells with lineage plasticity, our data suggest that ADRB2 depletion induces resistance to androgen depletion and antiandrogens, and possibly also EMT. High ADRB2 levels may promote the maintenance of epithelial characteristics and treatment-induced lineage plasticity.

We observed that patients experiencing a deferred onset of ADT experienced shorter times to CRPC. Although previous studies have shown conflicting findings as to whether time from RP to ADT associates with expedited or deferred CRPC development [390, 391], we found that administration of salvage bicalutamide associated with deferred ADT. While this was not surprising, our analysis showed that salvage bicalutamide also associated with expedited CRPC in men with HNPC. This finding indicated that bicalutamide may have prompted cross-resistance towards ADT. Although we did not establish a mechanistic explanation, durable bicalutamide administration to LNCaP cells has been shown to induce resistance mechanisms associated with PSA-negativity that may occur in clinical CRPCs [392]. Cyclic AMP signaling increases levels of the neuropeptide neurotensin [213] which potentiates isoproterenol responses in a feed-forward manner [393]. Although we did not directly investigate the association between ADRB2 and neurotensin, bicalutamide-resistant LNCaP cells have been reported to upregulate neurotensin mRNA and protein levels [394]. Thus, the notion that antiandrogens may prime tumors to resist ADT is likely true and may involve the β-adrenergic

signaling axis. Another possible explanation may relate to the bicalutamide withdrawal syndrome [112, 113], in which prolonged bicalutamide exposure may prompt AR mutations that turn bicalutamide into an AR agonist. Finally, bicalutamide may have lowered ADRB2 protein levels in residual tumor cells prior to ADT [255], resulting in priming of the tumors to resist the treatment.

8.7. Conclusion

In conclusion, the presented work has shown that prostate cancer tumors expressing low ADRB2 levels resist androgen-targeted therapy through reactivating AR signaling upon treatment challenge (Figure 8.1). Low-ADRB2 tumors thus represent a model for adenocarcinoma-CRPC. Upon androgen depletion, low-ADRB2 cells lack the β-adrenergic input to undergo androgen depletion-induced NEtD. Meanwhile, hormone-naïve high-ADRB2 tumors are characterized by androgen signaling and luminal differentiation. Like upon β-adrenergic stimulation, androgen depletion confers NEtD in high-ADRB2 cells. Thus, the expression level of ADRB2 in hormone-naïve tumors may be decisive of lineage fate following androgen-targeted therapies.

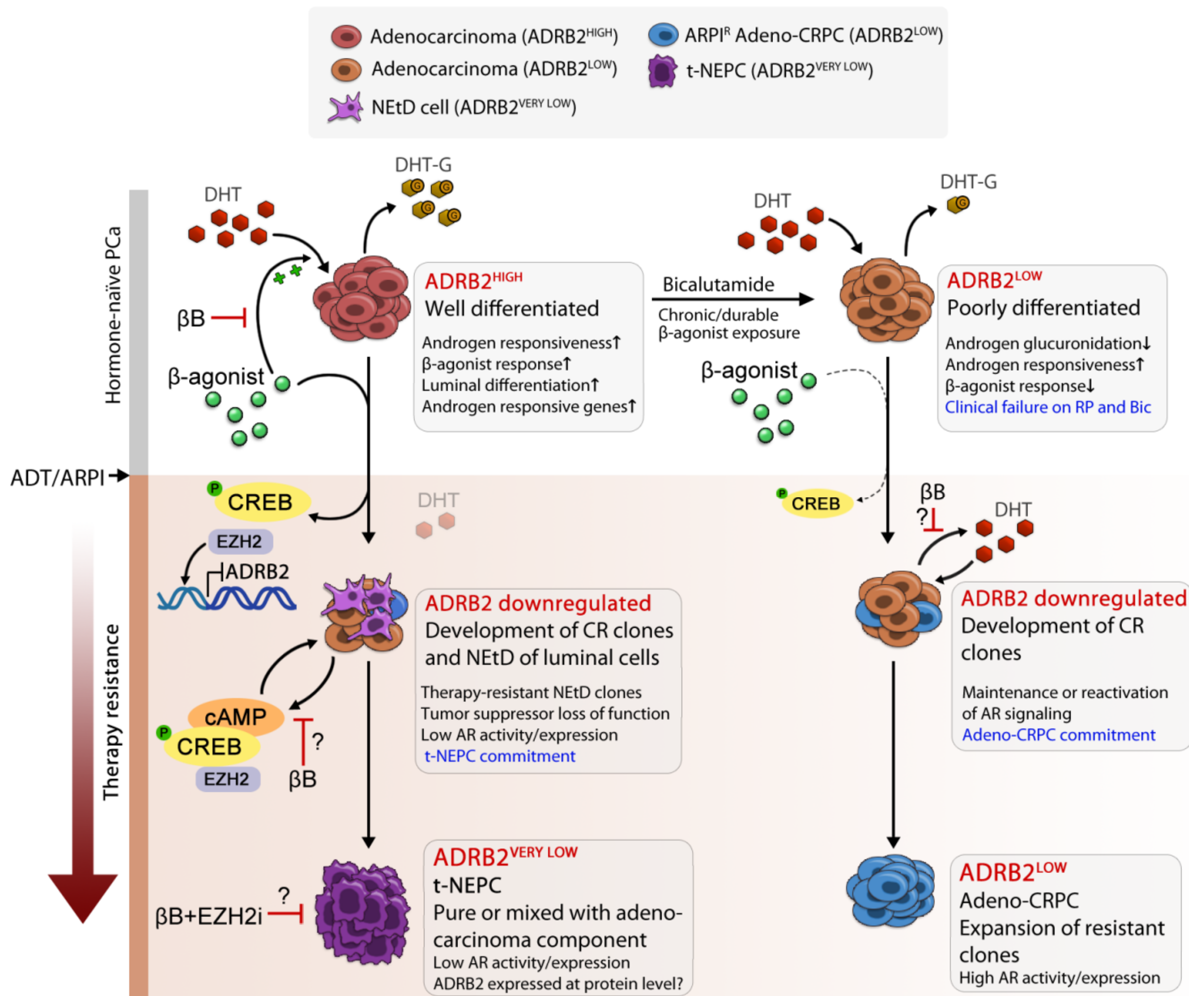


Figure 8.1. ***Hypothetical model for the differential effects of low- and high-ADRB2 expressing prostate cancer tumors undergoing androgen-targeted therapies.*** *High-ADRB2 expressing prostate cancers show a more differentiated, luminal growth pattern, and are responsive to sympathetic stimulation and thereby androgens via AR transactivation. Upon androgen-targeted therapy, these tumors lose AR signaling and undergo NEtD with subsequent transcriptional downregulation of ADRB2, potentially via ADRB2-driven EZH2 activation. NEtD cells may under further selective pressure give rise to t-NEPCs (pure or amphicrine). Low-ADRB2 expressing cells may be present in hormone-naïve prostate cancers or as a result of antiandrogen treatment or chronic exposure to ADRB-agonists. While these tumors do not respond to sympathetic stimuli, they have increased steroid biosynthesis and readily glucuronidate bioavailable androgens when challenged with androgen deprivation. These cells are thus primed to resist ADT by reactivation of the AR signaling axis, yielding CRPC-adenocarcinomas. The figure is in part adapted from [395]. βB = beta-blocker, DHT-G = glucuronidated DHT.*

9. Future directions, clinical utility and impact

The possibility of targeting β_2-adrenergic receptors with β-blockers is compelling since these drugs are off-patent, low cost and well tolerable. As incidental β-blocker use associates with favorable outcomes in prostate cancer patients, the presented findings add to the scientific debate about the underlying mechanisms explaining their potential molecular effects on prostate cancer cells. As we show that differential levels of ADRB2 associated with distinct resistance mechanisms to AR-targeted therapies, our findings may contest a general advice to offer beta-blockers to prostate cancer patients regardless of e.g. hormone-naivety and tumoral ADRB2 levels. We hypothesize that β-blockade may be most beneficial to prevent sympathetic bursts occurring during AR-targeted therapies, which in turn leads to ADRB2 desensitization and downregulation.

The question of whether reprogramming of AI-CRPCs to a less aggressive disease state is possible was recently asked [159]. The strong genomic and transcriptional rewiring observed in these tumors indicates that epigenetic drugs may have a clinical utility. Both β-adrenergic stimulation, androgen-targeted therapies and resistance to these treatments confer ADRB2 mRNA downregulation. ADRB2 downregulation is particularly apparent in t-NEPCs, which indicates that epigenetic silencing of ADRB2 is a determinant event in the emergence of this lethal prostate cancer variant. Furthermore, β-blockers may prevent β-adrenergic-driven ADRB2 downregulation. We are therefore aiming to investigate whether β-blockers, possibly in combination with an inhibitor of the BET family of bromodomains or EZH2 inhibitors, can prevent lineage plasticity and t-NEPC development in relevant model systems. It would also be of interest to retrospectively couple registry data on β-blocker use to patient cohorts containing patients who developed CRPC adenocarcinoma, t-NEPC or other AI-CRPC subtypes.

We are currently designing a pipeline in which we will assess whether ADRB2 is indeed expressed at the protein level in CRPCs and t-NEPC. This knowledge will be instrumental before initiating preclinical studies evaluating the efficacy of β-blockers in these disease stages. Ultimately, these studies can lay the ground for clinical trials that can help identify patient groups that will benefit from taking β-blockers alongside treatments currently used in clinical practice.

While a functional role of ADRB2 in the development of NEtD was established, we did not investigate whether a causal relationship between ADRB2 and t-NEPC development exists. With access to longitudinally matched tumor samples from hormone-naïve and preferably ADT + ARPI-treated prostate cancers, a more clear link may be determined. We are planning to investigate whether high- and low-ADRB2 LNCaP cells and xenografted tumors with loss-of-function alterations in *RB1* and/or *TP53* respond differentially upon AR-targeted combination therapies. Specifically, we hypothesize

that successful NEtD in high-ADRB2 cells will manifest as t-NEPC upon loss of tumor suppressor function. Furthermore, we plan to overexpress ADRB2 in the NEPC cell line NCI-H660, which presents strong ADRB2 downregulation, to investigate whether this can rewire the cells towards a more luminal-differentiated phenotype.

We are in the early phase of performing cDNA microarray analyses on hormone-naïve tumor tissue from additional patients who later underwent ADT, which will serve as a validation cohort. In particular, our finding that salvage bicalutamide may have conferred cross-resistance to ADT warrants further investigations, as it may have implications for therapy options in men undergoing e.g. enzalutamide alone or in combination with ADT for HNPC. This should preferably be investigated in a retrospective cohort that is more balanced in terms of salvage bicalutamide use.

The findings presented in this thesis paint an intricate and contextual relationship between the β_2-adrenergic and AR signaling cascades in prostate cancer. As a prognostic biomarker, low ADRB2 levels associated with expedited therapy resistance towards AR-targeted therapies. George Kulik recently suggested that tumors not responsive to β-blockers may rely on pathways such as the PI3K/AKT pathway [396], which falls in line with our unpublished data showing higher levels of phosphorylated Akt in low-ADRB2 LNCaP cells. Hence, while hormone-naïve patients with low tumoral ADRB2 levels may benefit from e.g. Akt-inhibitors, high-ADRB2 tumors may be more responsive to β-blockade and AR-targeted therapies.

Our findings have added onto the understanding of the role of ADRB2 in prostate cancer and has laid the ground for further studies on the elusive roles of this receptor. The presented findings display that ADRB2 may be an actionable target across various disease stages, and may aid in stratifying patients into treatment groups based on tumoral ADRB2-levels.

10. References

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I

Low β_2-adrenergic receptor level may promote development of castration resistant prostate cancer and altered steroid metabolism

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ABSTRACT

The underlying mechanisms responsible for the development of castration-resistant prostate cancer (CRPC) in patients who have undergone androgen deprivation therapy are not fully understood. This is the first study to address whether β_2-adrenergic receptor (ADRB2)- mediated signaling may affect CRPC progression *in vivo.* By immunohistochemical analyses, we observed that low levels of ADRB2 is associated with a more rapid development of CRPC in a Norwegian patient cohort. To elucidate mechanisms by which ADRB2 may affect CRPC development, we stably transfected LNCaP cells with shRNAs to mimic low and high expression of ADRB2. Two UDP-glucuronosyltransferases, UGT2B15 and UGT2B17, involved in phase II metabolism of androgens, were strongly downregulated in two LNCaP shADRB2 cell lines. The low-ADRB2 LNCaP cell lines displayed lowered glucuronidation activities towards androgens than high-ADRB2 cells. Furthermore, increased levels of testosterone and enhanced androgen responsiveness were observed in LNCaP cells expressing low level of ADRB2. Interestingly, these cells grew faster than high-ADRB2 LNCaP cells, and sustained their low glucuronidation activity in castrated NOD/

SCID mice. ADRB2 immunohistochemical staining intensity correlated with UGT2B15 staining intensity in independent TMA studies and with UGT2B17 in one TMA study. Similar to ADRB2, we show that low levels of UGT2B15 are associated with a more rapid CRPC progression. We propose a novel mechanism by which ADRB2 may affect the development of CRPC through downregulation of UGT2B15 and UGT2B17.

INTRODUCTION

Androgen deprivation therapy (ADT) is the first line of treatment for patients with advanced or metastatic prostate cancer [1]. ADT is initially effective in controlling tumor growth and symptoms, but most tumors eventually develop resistance to ADT and become castration resistant prostate cancers (CRPC). Over the last years, it has become evident that the androgen signaling axis plays a pivotal role in the development of CRPC [2]. The multiple molecular mechanisms by which the androgen receptor (AR) contributes to disease progression despite castration levels of androgens in prostate cancer have been thoroughly reviewed [3-6]. Several new targets in the AR activation pathway have emerged in recent years [7, 8]. The steroidogenic pathway has received increasing attention, as drugs targeting this pathway, such as abiraterone (an inhibitor of cytochrome P450, family 17, subfamily A, polypeptide 1 (CYP17)) improve the life expectancy of patients with CRPC, despite the assumed androgen-independence of these cancer cases [8]. No curative options for CRPC are, however, available today. Increased knowledge of the mechanisms by which the cancer cells progress to CRPC is hence needed. Recently, targeting the androgen extrahepatic phase-II metabolic pathways has arisen as a potential tool to help maintain androgen-deprived conditions during ADT [9]. The UDP-glucuronosyltransferases 2B15 (UGT2B15) and 2B17 (UGT2B17) are of special interest, as they are expressed in prostate tissue and cell lines, and they exhibit specificity for androgen metabolites [10].

The β_2-adrenergic receptor (ADRB2) and its downstream effectors cyclic AMP (cAMP) and cAMP-dependent protein kinase A (PKA) have been implicated in prostate cancer progression and AR signaling [11]. In particular, sympathetic stimulation of ADRB2 has been shown to potentially sensitize AR in cell lines under androgen depleted conditions [12], suggesting that ADRB2 might play a role in the development of CRPC. Furthermore, a number of target genes are common for the androgen and the PKA signaling cascades [13], and in steroidogenic cells both cAMP and PKA have been shown to regulate transcription of steroidogenic genes such as CYP17 and STAR [14-16], as well as to modulate their activity at the protein level [17].

While most pre-clinical evidence points towards a tumor promoting role of β-adrenergic signaling [18, 19], a previous study by Yu et al. reported an inverse correlation between ADRB2 expression levels and prostate cancer progression [20]. Low levels of ADRB2 in prostate cancer tissue were found to correlate with biochemical recurrence measured as increasing prostate-specific antigen (PSA) levels, or metastatic disease after radical prostatectomy. Conversely, our group has recently reported an association between the use of β-blockers (ADRB antagonists) and improved prostate cancer specific survival both for patients who have undergone ADT [21] and for patients with high risk or metastatic disease [22].

Our knowledge about the potential role of the ADRB2 in prostate cancer and CRPC development is still limited. Therefore, in this study, we have addressed this topic by performing immunohistochemical analyses and investigated the potential role of ADRB2 in development of CRPC in ADRB2 knockdown cell lines.

RESULTS

Low ADRB2 expression level in tumor tissue is associated with poor prognosis after androgen deprivation therapy

Tissue from 45 prostate cancer patients who had received hormonal therapy and had been treated with transurethral resection of the prostate (TUR-P) at Oslo University Hospital, Aker (the Oslo ADT cohort) were included in a tissue micro-array study. Five patients were excluded due to lack of cancerous tissue following staining with anti-ADRB2 antibody. The mean follow-up from initiation of ADT for the 40 patients included in the survival analyses was 71 months. For prostate cancer-specific mortality the mean follow-up was 70 months, as we lacked information on the cause of death for four patients. Patient and tumor characteristics at time of diagnosis are shown in Supplementary Table 2. Examples of negative and strong ADRB2 staining of two specimens with Gleason score 9 are shown in Figure 1a and 1b. Kaplan-Meier plots showing time to CRPC development and prostate cancer- specific mortality in patients stratified according to staining intensity above and below mean are shown in Figure 1c and 1d. Competing risk regression modelling showed that increasing staining intensity was associated with increased time to CRPC development, with an adjusted SHR of 0.67 (95% CI 0.46-0.97, *p*-value 0.035; adjusted for age at initiation of ADT and Gleason score) (Table 1). For prostate cancer- specific mortality, the association was not statistically significant (adjusted

Table 1: Uni- and multivariable HRs/SHRs for ADRB2 staining intensity and CRCP development and prostate cancer-specific and all-cause mortality.

	Cumulative incidence	Increasing ADRB2 staining intensity	
		Crude estimate SHR/HR (95 % CI) *p*-value	Multivariable analysis[a] SHR/HR (95% CI) *p*-value
Development of CRPC[b]	27/40	0.77 (0.53-1.13) 0.18	0.67 (0.46-0.97) 0.035
Prostate cancer- specific mortality[b]	21/35	0.71 (0.47-1.08) 0.11	0.70 (0.42-1.15) 0.16
Overall mortality	36/40	0.74 (0.53-1.04) 0.082	0.91 (0.61-1.37) 0.66

[a] Adjusted for age at initiation of androgen deprivation therapy and highest Gleason score from HE-slides of the TMA
[b] Analyzed by competing risk regression

SHR 0.70, 95% CI 0.42-1.15, *p*-value 0.16). ADRB2 levels had no impact on all-cause mortality (adjusted HR 0.91, 95% CI 0.61-1.37, *p*-value 0.66).

A correlation analysis indicated no association between ADRB2 expression level and duration of ADT before TUR-P surgery (correlation coefficient -0.21, *p*-value 0.23).

LNCaP shADRB2-tumors grow more rapidly in castrated mice

Aiming to reveal potential mechanisms explaining the observed correlation between ADRB2 expression and time to CRPC development, we stably transfected LNCaP cells with shRNA plasmids targeting ADRB2 mRNA, yielding two knockdown cell lines (shADRB2-1

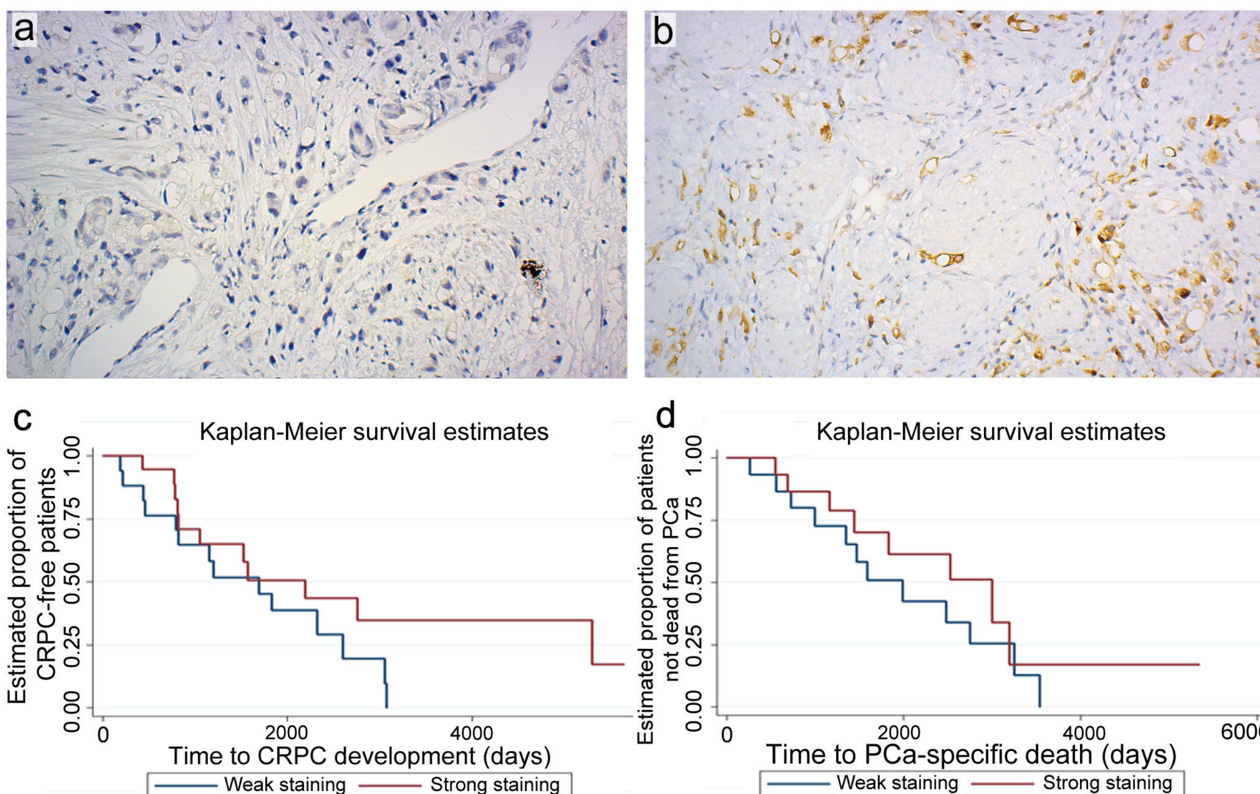

Figure 1: ADRB2 level is positively correlated with time to CRPC development. Immunohistochemical analysis of ADRB2 expression in a TMA of transurethral resections of the prostate (TUR-P). Examples of tissue cores of Gleason score 9 tumors showing negative **a.** or strong staining **b.** intensity (original magnification 20x). Kaplan-Meier plots showing time to CRPC development **c.**, and time to prostate cancer (PCa)- specific death **d.** following TUR-P in patients stratified according to strong and weak staining intensity of ADRB2.

and 2), as well as a non-targeting shRNA plasmid (shCtrl). Real-Time RT-PCR analyses on mRNA isolated from shADRB2 and shCtrl cells revealed a 50% and 95% reduction of ADRB2 mRNA in shADRB2-1 and shADRB2-2, respectively, compared to shCtrl (Figure 2a). Radiolabeled ligand-binding assay measuring ^{125}I-cyanopindolol (CYP)-binding to membrane-bound ADRBs confirmed the knockdown, with 50% and 85% lowered ADRB binding activity in shADRB2-1 and shADRB2-2 cells, respectively (Figure 2b). The receptor acts primarily through stimulating adenylyl cyclase (AC) activity, resulting in increased cAMP levels. The basal (non-stimulated) rate of conversion of [α-^{32}P]ATP to [^{32}P] cAMP was significantly lowered in both shADRB2-1 and 2 as shown in Figure 2c. Furthermore, stimulation with the non-selective ADRB-agonist isoproterenol showed a larger absolute and relative increase in adenylyl cyclase activity in shCtrl compared to both shADRB2 cell lines, indicating a functional effect of reduced ADRB2 levels.

LNCaP shADRB2-2 and shCtrl cells were injected into NOD-SCID mice. The mice were castrated when the tumor diameter reached 10-12 mm and the tumor growth was followed in castrated mice for up to 42 days. After a brief lag period, the shADRB2-2 tumors grew more rapidly after castration, as shown in Figure 3a. Although the ten mice in the shADRB2-2 group had non-significantly smaller tumors than the eleven mice in the shCtrl group at time of castration, the shADRB2-2 tumors were larger 28 days after castration. The change in tumor volume from day 0 to day 42 was 3.5 fold higher in the shADRB2-2 compared to the shCtrl group (Figure 3b).

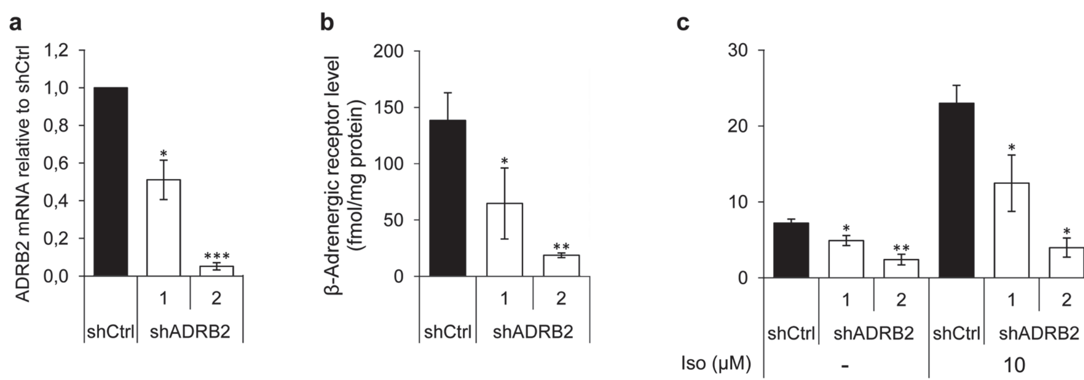

Figure 2: ADRB2 level, receptor binding, and downstream signaling activity in LNCaP shADRB2 cell lines. a. ADRB2 mRNA levels were semi-quantitatively measured in RNA isolated from two LNCaP shADRB2 (shADRB2-1 and shADRB2-2) cell lines and a non-targeting shRNA LNCaP cell line (shCtrl) using Real-Time RT-PCR. Mean, $\Delta\Delta C_t$ calculated values relative to shCtrl cells are shown. **b.** β-adrenergic receptor level was quantified by determination of ^{125}I-CYP specific binding to membrane protein fractions isolated from two LNCaP shADRB2 cell lines and shCtrl cells. Bars represent β-adrenergic receptor level reported as fmol/mg protein in the membrane fraction. **c.** Adenylyl cyclase activities in membranes isolated from LNCaP shADRB2 and shCtrl cells treated with vehicle or 10 μM isoproterenol were measured. The bars represent mean rate of formation of cAMP normalized to total protein in the membrane fractions (fmol/mg protein/min). All experiments were performed in biological triplicates (n = 3), mean ± standard deviation (SD). Statistical significance is indicated by asterisks (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

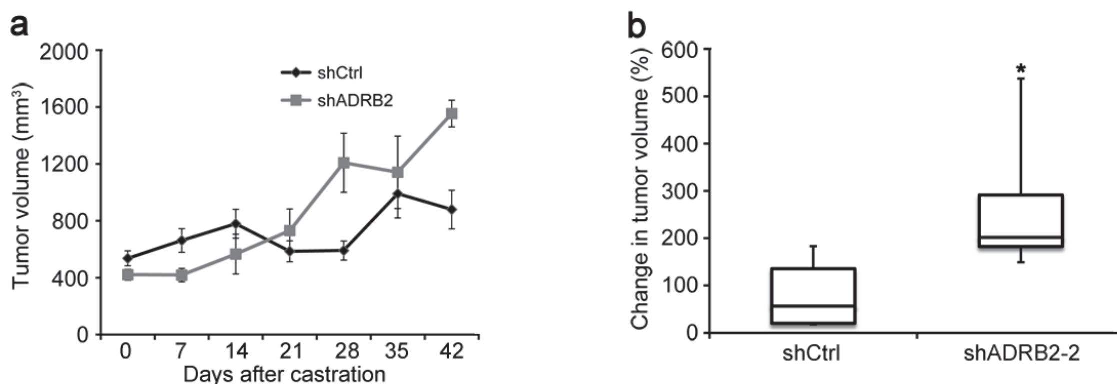

Figure 3: LNCaP shADRB2 xenograft tumors grow more rapidly than shCtrl tumors in castrated mice. LNCaP shADRB2-2 and shCtrl cells were implanted subcutaneously into nude NOD-SCID mice. Once tumors reached 500 mm^3 in size, mice were surgically castrated and taken off testosterone supplementation. Tumor volumes were measured weekly for 6 weeks. The graph **a.** shows mean (n = 10 for shADRB2-2 and 11 for shCtrl) tumor volumes (mm^3) ± SEM. **b.** Box-and-whisker plot showing the percentage change in tumor volume 42 days after castration in NOD-SCID mice injected with LNCaP shADRB2-2 and shCtrl cells. Statistical significance was measured by Fischer exact test, and is indicated by asterisks (*: $p < 0.05$).

Table 2: Spearman's rank correlations between ADRB2 and UGT2B15 and UGT2B17

		ADRB2 versus UGT2B15		ADRB2 versus UGT2B17	
TMA study	**Cohort**	**No. of pairs**	**Correlation (95% CI) *p*-value**	**No. of pairs**	**Correlation (95% CI) *p*-value**
Oslo ADT		65	0.39 (0.16-0.59) 0.001	64	0.19 (-0.066-0.42) 0.13
Vancouver Prostate Centre Tissue Bank	All cancer cases	583	0.40 (0.33-0.47) <0.0001	602	0.35 (0.27-0.42) <0.0001
	Recurrent PCa	209	0.50 (0.38-0.59) <0.0001	214	0.33 (0.20-0.45) <0.0001
	CRPC	58	0.64 (0.45-0.78) <0.0001	58	0.33 (0.074-0.55) 0.011

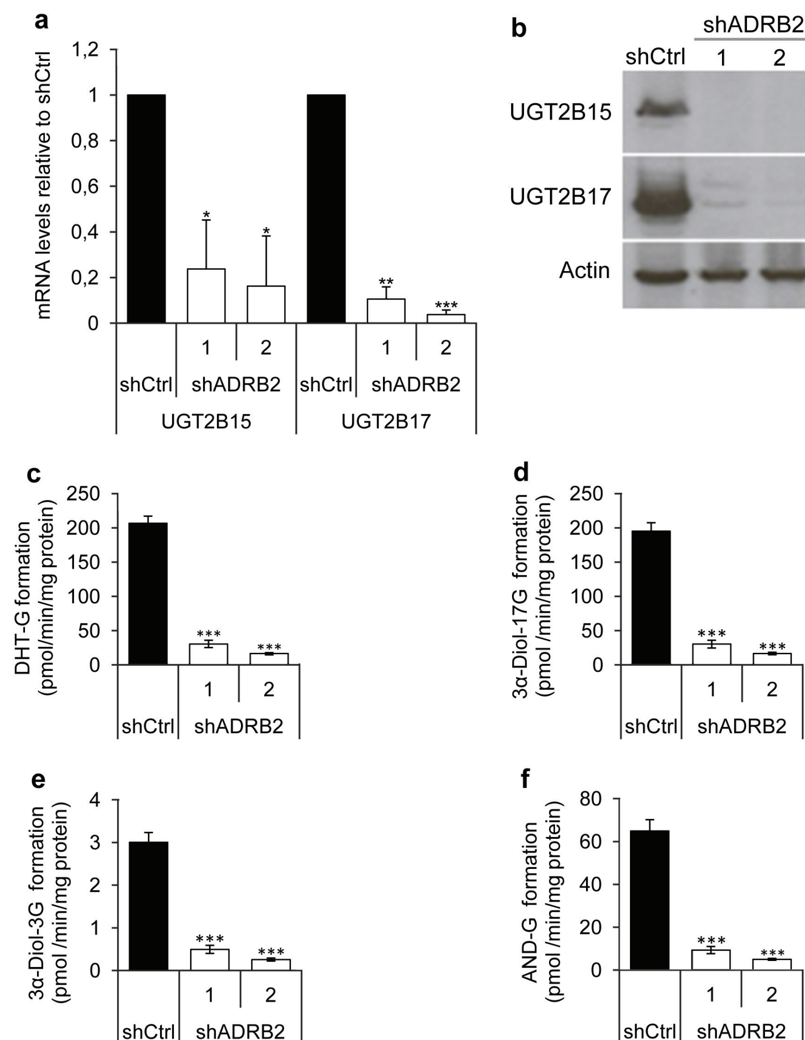

Figure 4: UGT2B15 and UGT2B17 mRNA, protein and effects on androgen glucuronide formation. a. UGT2B15 and UGT2B17 mRNA levels were measured in RNA isolated from LNCaP shADRB2 (shADRB2-1 and shADRB2-2) and shCtrl cells using Real-Time RT-PCR. Bars represent mean, $\Delta\Delta C_t$ calculated values relative to shCtrl cells ($n = 3$) ± SD. **b.** UGT2B15 and UGT2B17 protein levels were visualized in cell homogenates by immunoblotting using anti-UGT2B15 and anti-UGT2B17 antibodies. Anti-actin antibodies were simultaneously used on the same homogenates to ensure similar loading on the lanes. **c.-f.** Cell homogenates from two LNCaP shADRB2 cell lines (shADRB2-1 and shADRB2-2) and shCtrl LNCaP cells (shCtrl) were mixed with uridine diphosphate glucuronic acid (UDPGA) and either dihydrotestosterone (DHT), 3α-androstanediol (3α-Diol) or androsterone (AND), for one hour, and levels of glucuronidated (G) androgens (c: DHT-G; d: 3α-Diol-17G; e: 3α-Diol-3G; f: AND-G) were measured by LC-MS/MS. The results are shown as mean formed glucuronide related to total protein in the homogenates (pmol/min/mg protein) from duplicated reactions on three biological replications ± SD. Statistical significance is indicated by asterisks (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Table 3: A) Uni- and multivariable HRs/SHRs for UGT2B15 staining intensity and CRCP development and prostate cancer- specific and all-cause mortality. B) Uni- and multivariable HRs/SHRs for UGT2B17 staining intensity and CRCP development and prostate cancer- specific and all-cause mortality.

A)	**Cumulative incidence**	**Increasing UGT2B15 staining intensity**	
		Crude estimate SHR/HR (95 % CI) *p*-value	**Multivariable analysisa SHR/HR (95% CI) *p*-value**
Development of CRPC [b]	22/33	0.63 (0.32-1.25) 0.19	0.39 (0.16-0.97) 0.043
Prostate cancer- specific mortality[b]	15/28	0.63 (0.30-1.32) 0.22	0.38 (0.09-1.59) 0.19
Overall mortality	29/33	0.67 (0.37-1.22) 0.19	0.90 (0.42-1.95) 0.80
B)	**Cumulative incidence**	**Increasing UGT2B17 staining intensity**	
		Crude estimate SHR/HR (95 % CI) *p*-value	**Multivariable analysisa SHR/HR (95% CI) *p*-value**
Development of CRPC [b]	23/34	1.06 (0.55-2.05) 0.87	0.87 (0.43-1.73) 0.69
Prostate cancer- specific mortality[b]	16/29	0.89 (0.46-1.71) 0.72	0.69 (0.41-1.16) 0.16
Overall mortality	30/34	0.93 (0.52-1.67) 0.80	1.17 (0.60-2.30) 0.65

[a] Adjusted for age at initiation of androgen deprivation therapy and highest Gleason score from HE-slides of the TMA
[b] Analyzed by competing risk regression

Knockdown of ADRB2 in LNCaP cells is associated with reduced androgen glucuronidation activity

We performed gene expression profiling of the LNCaP shADRB2 and shCtrl cells to aid in elucidating potential mechanisms explaining the association between ADRB2 and CRPC development, as well as the increased growth of the shADRB2 xenograft tumors. From this microarray analysis we observed differential expression of UDP-glucuronosyltransferase 2B15 and 2B17 in shADRB2 cells compared to the shCtrl cells (data not shown). To corroborate the microarray data, we performed Real-Time RT-PCR which showed that UGT2B15 was down-regulated 5-fold and 6-fold, and UGT2B17 down-regulated 10-fold and 20-fold, in shADRB2-1 and 2 respectively, relative to shCtrl (Figure 4a). The UGT2B15 and UGT2B17 protein levels were visualized by immunoblotting analysis. Whereas both proteins showed strong bands in shCtrl cells, UGT2B15 and UGT2B17 were virtually un-detectable in both shADRB2 cell lines (Figure 4b).

Furthermore, lowered UGT2B15 and UGT2B17 expression was accompanied by reduced androgen glucuronide formation (Figure 4c-4f). Dihydrotestosterone-glucuronide (DHT-G), two androstanediol glucuronides (3α-Diol-17G, 3α-Diol-3G) and androsterone glucuronide (AND-G) formation was strongly reduced in the shADRB2 cell lines compared to shCtrl cells, with a steady 85% lowering of glucuronide formation in shADRB2-1 cells, and a 95% fold lowering in shADRB2-2 cells. Glucuronidation activity in positive (human liver homogenates) and negative (HEK293 cell homogenates) controls is shown in Supplementary Figure 1.

These findings led us to investigate whether castration of mice injected with LNCaP shCtrl or shADRB2-2 cells had an effect on the expression and activity of UGT2B15 and UGT2B17 *in vivo*. Immunohistochemical staining of tumor tissue from the xenograft study using anti-UGT2B15 and anti-UGT2B17 antibodies showed that the phenotypic differences between shCtrl and shADRB2-2 cells were maintained also after castration (Figure 5a). UGT2B15 and UGT2B17 staining intensities were statistically significantly higher in shCtrl tumors than shADRB2-2 tumors (p = 0.006 and p = 0.0004 for UGT2B15 and UGT2B17, respectively). UGT2B17 negatively correlated to average daily growth of the tumors (correlation coefficient -0,518, p = 0.016), whereas UGT2B15 did not (correlation coefficient -0.188, p = 0.41). Furthermore, the glucuronidation activity in tumor extracts was on average 85% lower in shADRB2 xenograft mice compared to shCtrl mice (Figure 5b-5e).

Knockdown of ADRB2 improves androgen responsiveness *in vitro*

After confirming that lowered ADRB2 expression lead to a change in glucuronidation activity, we were interested in finding out whether this could provoke a

change in the AR activity in the cells. DHT-stimulated LNCaP shADRB2 and shCtrl cells transiently transfected with the probasin-based promoter and luciferase reporter construct 285-Pb-pEZX-PG04 revealed that shADRB2 cells had a higher relative androgen responsiveness than shCtrl cells (7-fold and 4-fold in shADRB2-1 and 2, respectively) (Figure 6a). Similar results were obtained when the cells were pre-incubated for 96 hours in hormone-deprived medium prior to stimulation (data not shown). To test if the effect was caused by reduced level of ADRB2, we rescued ADRB2 expression in shADRB2-1 and 2 using pCDNA3.1-ADRB2, constitutively expressing the ADRB2 gene. The relative luciferase activity was decreased by 80% and 50%, yielding the knockdown cells

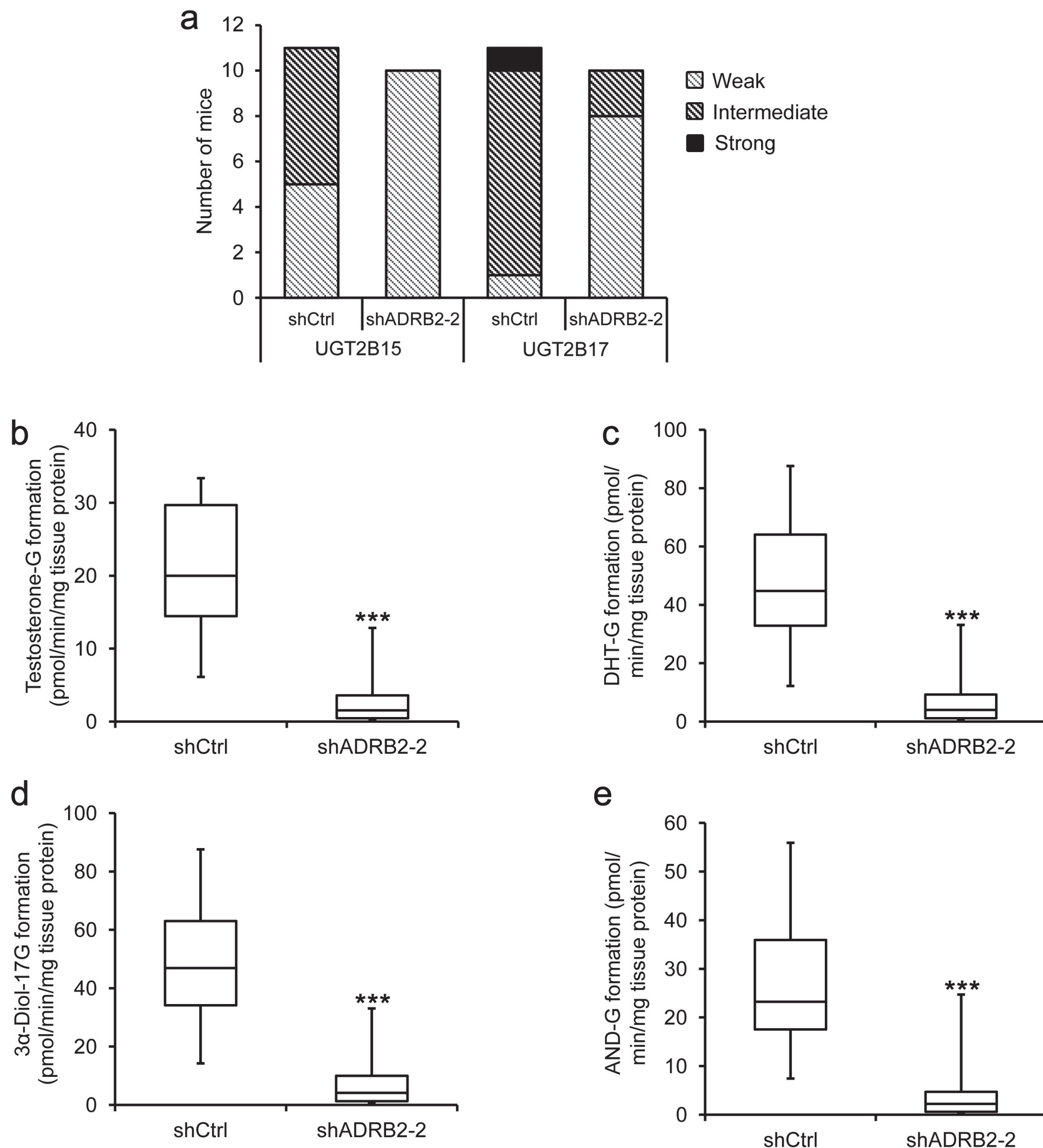

Figure 5: LNCaP shADRB2 castrated mouse tumor characteristics and glucuronidation activity. a. Excised xenograft tumors were formalin-fixed and paraffin-embedded, and sections were stained with anti-UGT2B15 (1:500) and anti-UGT2B17 (1:500) antibodies. Frequencies of staining intensities (weak, intermediate and strong) from tumors derived from mice injected with shCtrl (n = 11) and shADRB2-2 (n = 10) LNCaP cells are shown. **b.-e.** Fresh frozen tumor tissue from the same mice were homogenized and added UDPGA and either dihydrotestosterone (DHT), 3α-androstanediol (3α-Diol) or androsterone (AND) in a glucuronidation assay. Formation of steroid glucuronides (c: DHT-G; d: 3α-Diol-17G; e: 3α-Diol-3G; f: AND-G) was measured by LC-MS/MS. The results are shown as a box-and-whisker plot showing formed glucuronide related to total protein in the tissue homogenates (pmol/min/mg tissue protein) from triplicate reactions on the same homogenates. Statistical significance is indicated by asterisks (**: $p < 0.01$; ***: $p < 0.001$).

more similar to shCtrl cells (Figure 6b). Furthermore, we wanted to test if the effect observed with a probasin-based promoter (285-Pb-pEZX-PG04) could be replicated with a different androgen responsive reporter plasmid containing 7 kb of the 5' upstream region of the PSA promoter (pGL3/PSA). We transfected shADRB2 and shCtrl cells with pGL3/PSA and measured relative androgen responsiveness after stimulation with 10 nM DHT or vehicle for 48 hours. As with 285-Pb-pEZX-PG04, the androgen response of pGL3/PSA was higher when transfected into shADRB2 cells than shCtrl cells (2.5- and 1.8-fold higher in shADRB2-1 and shADRB2-2, respectively) (Figure 6c).

Next, we were interested in seeing whether the androgen responsiveness could be modulated by inhibiting UGT2B15 and UGT2B17 activity. We treated LNCaP shCtrl cells with the UGT2B substrate diclofenac sodium (DFS), which competitively inhibits UGT2B-action towards androgens. Stimulation with 50 µM diclofenac sodium induced a statistically significant 1.8-fold rise in normalized 285-Pb-pEZX-PG04-driven luciferase activity (Figure 6d).

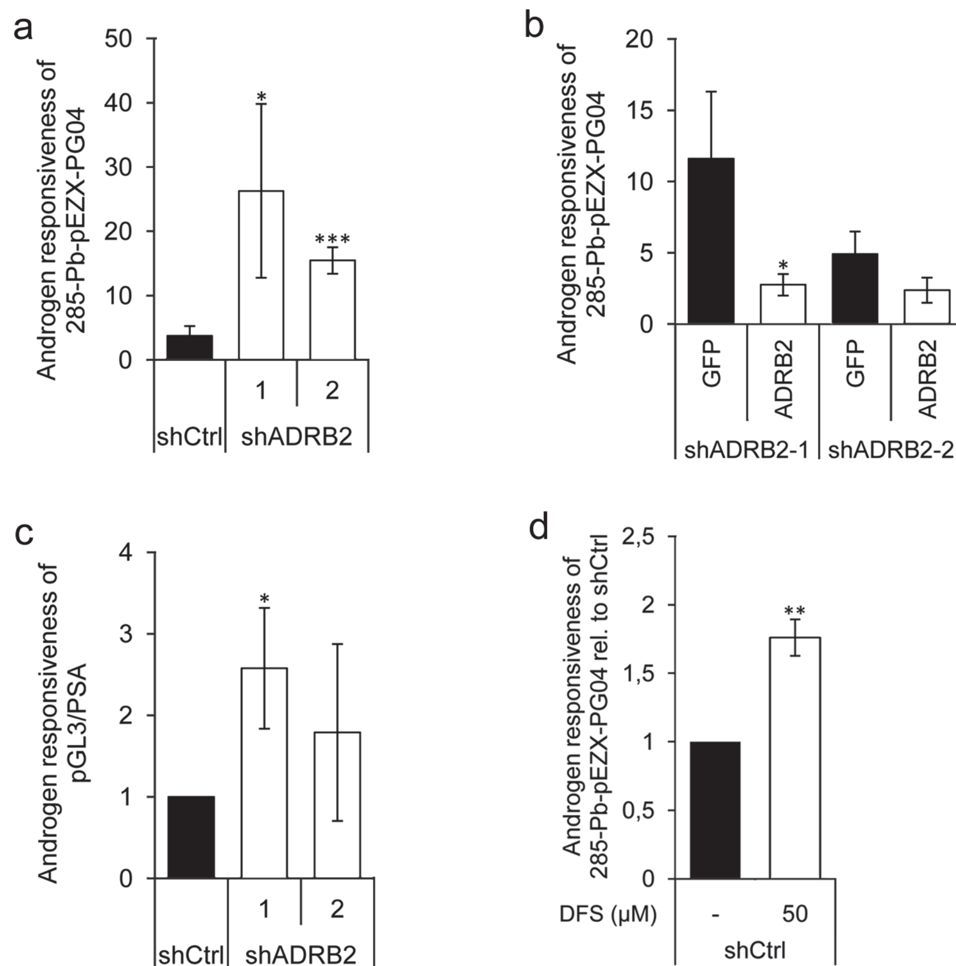

Figure 6: Increased androgen responsiveness in LNCaP shADRB2 cell lines. a. LNCaP shADRB2-1, shADRB2-2 and shCtrl cells were transfected with the androgen responsive element-containing luciferase reporter construct 285-Pb-pEZX-PG04. The following day, cells were incubated in hormone-deprived medium containing 2% CSS supplemented with either 10 nM DHT or vehicle and further incubated for 48 hours. The androgen responsiveness of 285-Pb-pEZX-PG04 in LNCaP shADRB2-1, shADRB2-2, and shCtrl cells are shown relative to vehicle treated (n = 3) ± SD. . **b.** LNCaP shADRB2-1 and shADRB2-2 were transfected with the reporter construct 285-Pb-pEZX-PG04 and either an ADRB2 expression vector (pCDNA3.1-ADRB2) or a control expression vector (pEGFP-C3). Mean androgen responsiveness relative to vehicle treated cells is shown (n = 3) ± SD.. **c.** Cells were transfected with a reporter plasmid including the 5'-regulatory region of PSA (pGL3/PSA), and the cells were stimulated as described in (a). Androgen responsiveness is given as the relative luciferase activities from DHT-stimulated cells normalized to vehicle-treated cells from three independent experiments (n = 3) mean ± SD. **d.** shCtrl cells were transfected with 285-Pb-pEZX -PG04 and were either treated with 50 µM diclofenac or with vehicle and then half of the cells were stimulated with 10 nM DHT the following day and all cells were harvested after 72 hours. Mean DHT responses from three independent experiments are shown relative to un-stimulated shCtrl cells (given value 1.0) ± SD treated with either diclofenac or vehicle ± SD. Statistical significance is indicated by asterisks (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

The androgen responsiveness was non-significantly higher in the shADRB2-1 cell line than in the shADRB2-2 cell line (Figure 6a). This might be due to the fact that the androgen receptor is slightly induced in the shADRB2-1 cell line (measured by western immunoblotting of protein extracts; Supplementary Figure 2). The androgen receptor was not up-regulated in shADRB2-2 cells compared to shCtrl.

Prostate-specific antigen responsiveness is increased in ADRB2 knockdown LNCaP cells

We hypothesized that increased reporter-driven androgen responsiveness would be mirrored by an increase in the PSA response upon androgen stimulation. shADRB2 and shCtrl cells were pre-incubated in hormone-deprived medium for 96 hours, and then stimulated for 48 hours with either 1 nM DHT, 1 nM R1881, or vehicle, before harvesting and isolating total RNA. The Real-Time RT-PCR reaction revealed that DHT induced a significantly more pronounced response on PSA mRNA in both shADRB2 cell lines than in shCtrl (Figure 7a). Stimulation with the non-glucuronidable synthetic androgen R1881 resulted in a greater response in all the cells compared to DHT, but there were no significant differences between shADRB2 and shCtrl cells (Figure 7b). To substantiate these findings, we performed a similar experiment where we measured secreted PSA in medium from androgen-stimulated cells. Figure 7c shows the androgen responsiveness from cells stimulated with DHT, and Figure 7d shows the relative responses acquired with R1881. As with PSA mRNA, only stimulation with DHT yielded a significant difference in increased PSA-response between shADRB2 cell lines and the shCtrl cell line.

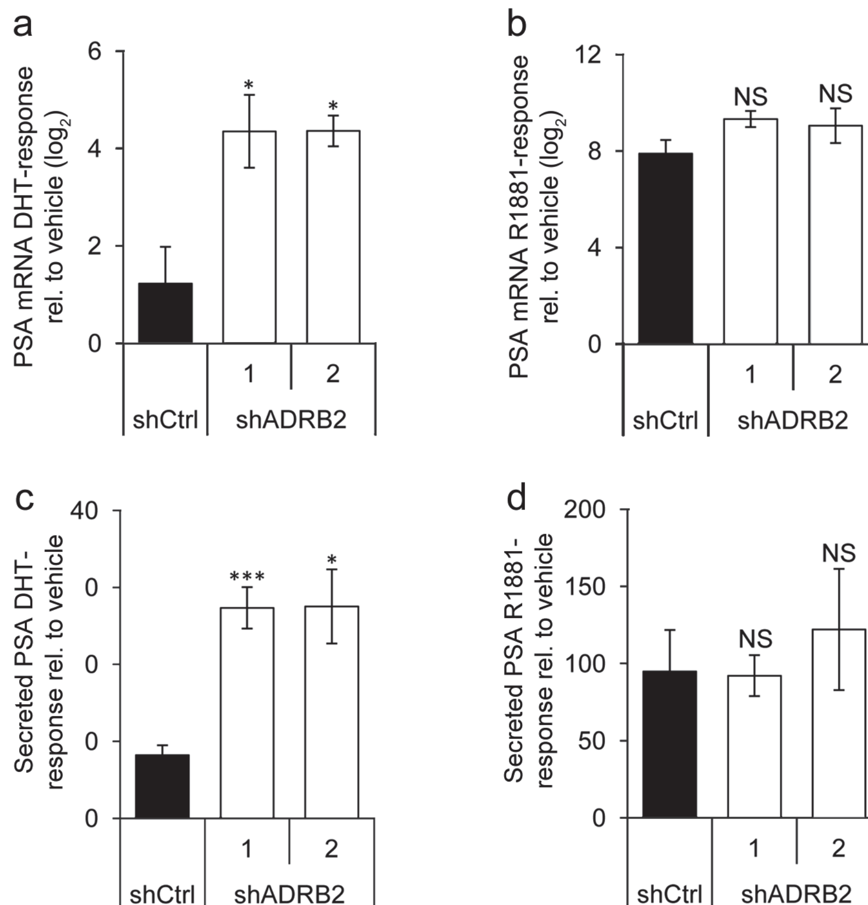

Figure 7: Prostate-specific antigen responsiveness is higher in shADRB2 than in shCtrl cells. **a.**, **b.** LNCaP shADRB2 and shCtrl cells were starved in 2% CSS for 96 hours prior to stimulation with 1 nM DHT or 1 nM R1881 for 48 hours. RNA was harvested and analyzed for PSA/KLK3 mRNA expression by Real-Time RT-PCR. Gene expression upon stimulation with **a.** DHT and **b.** R1881 relative to non-stimulated cells (vehicle) was calculated by the $\Delta\Delta C_t$- method. Bars represent log_2-transformed androgen responses ($n = 3$) ± SEM. **c.**, **d.** Secreted total PSA (TPSA) was measured in medium samples from cells stimulated with **c.** DHT and **d.** R1881 by time-resolved fluorescence, and was related to non-stimulated cells (vehicle). Bars represent androgen responses ($n = 4$) mean ± SD. NS: non-significant difference from shCtrl.

Glucuronidation activity affects androgen levels in LNCaP ADRB2 knockdown cells

A plausible effect of lowered glucuronidation activity is shifting of the substrate/glucuronide homeostasis and subsequent accumulation of glucuronidable androgens, which could help explain the observed increase in androgen responsiveness in shADRB2 cells. We therefore measured intracellular testosterone levels in shCtrl and shADRB2 cells cultured in FCS medium for 48 hours (Figure 8a). An 11-fold and 5.5-fold higher testosterone level was found in shADRB2-1 and shADRB2-2, respectively, compared to shCtrl. The basal reporter activity driven by the androgen responsive probasin promoter (pPB(-285/132)-LUC) was higher in shADRB2 cell lines compared shCtrl cells (Figure 8b) supporting that the testosterone level is increased in shADRB2 cell lines.

Furthermore, to establish a link between glucuronidation activity and levels of bioavailable androgen, we supplemented shCtrl and shADRB2-2 cells with diclofenac sodium and measured the intracellular testosterone levels. Diclofenac sodium caused a dose-dependent induction in testosterone levels, with 17-fold and 35-fold induction with 50 and 100 µM DFS, respectively (Figure 8c). Comparably, diclofenac had only a minor effect in shADRB2-2, which has 95% lower androgen glucuronidation activity than shCtrl cells.

UGT2B15 and UGT2B17 are correlated to ADRB2 in two patient material data sets

Initially, as we observed a regulation of UGT2B15 and UGT2B17 levels after ADRB2 knockdown *in vitro*, we wanted to examine if these proteins were correlated with ADRB2 expression in the Oslo ADT cohort. Both UGT2B15 and UGT2B17 predominantly showed cytoplasmic staining of luminal cells (Supplementary Figure 3). As can be seen in Table 2, UGT2B15 staining was found to be positively correlated with ADRB2 staining (correlation coefficient 0.39, *p*-value 0.001). A similar trend was found for UGT2B17; however this was not statistically significant.

To verify the results in an independent study, immunohistochemical stainings with ADRB2, UGT2B15 and UGT2B17 were performed on four TMAs, merged into a single data set, from the Vancouver Prostate Centre Tissue Bank. Of a total of 306 patients, 262 had cores positive for cancer. Among these, 96 patients had recurrent prostate cancer, 23 had diagnosed CRPC, and the remaining 143 were patients who have undergone radical prostatectomy. In this cohort, we observed a correlation between ADRB2 and UGT2B15 similar to the correlation in the Oslo ADT cohort (Table 2). Interestingly, the correlation was stronger when only samples from patients who have experienced recurrence or who have developed castration resistant prostate cancer were included in the analysis. In this dataset, the UGT2B17 staining was also significantly correlated with ADRB2 staining (Table 2).

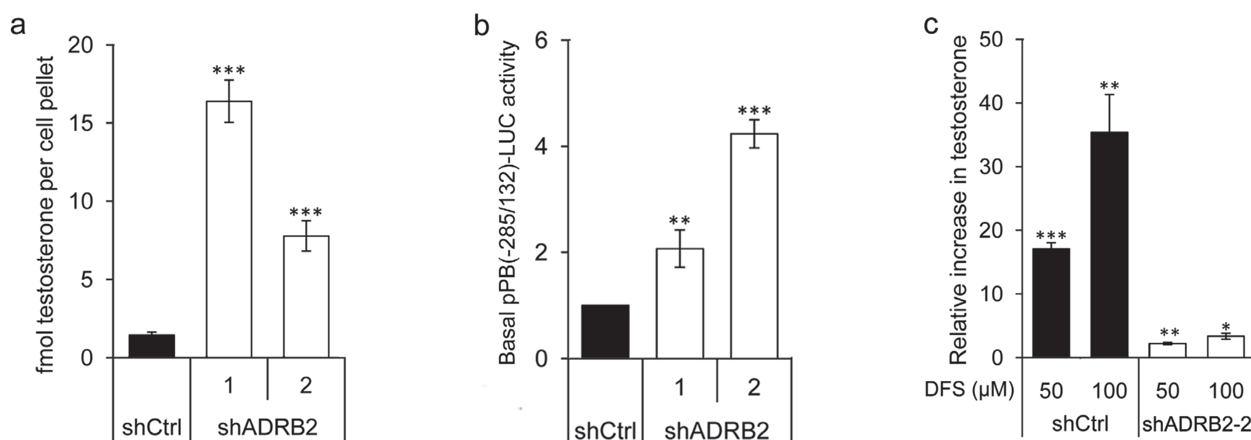

Figure 8: Reduced androgen glucuronidation activity affects the level of bioactive androgen *in vitro*. a. The basal level of testosterone was measured in shCtrl, shADRB2-1, and shADRB2-2 LNCaP cells cultured in FCS medium. Steroids were extracted from the cells, dried, reconstituted, and run on an LC-MS. Integrated, internal standard (IS)-normalized mean peak areas are shown related to equal cell pellets. **b.** LNCaP shADRB2-1, shADRB2-2 and shCtrl cells were transfected with pPB(-285/132)-LUC. After 72 hours incubation in FCS-medium, basal luciferase activity of the pPB(-285/132)-LUC reporter was measured and related to SEAP. The results are shown as mean, basal luciferase activities related to shCtrl (given value 1.0) ± SD from three independent experiments. **c.** LNCaP shADRB2-2 and shCtrl cells were treated with 50 or 100 µM diclofenac or vehicle for 48 hours. Integrated, internal standard (IS)-normalized peak areas were related to total protein content and is presented as mean relative increase compared to vehicle treated cells (given value 1.0) (nM testosterone/mg protein) from three independent experiments ± SD. Statistical significance is indicated by asterisks (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

Finally, we performed a competing risk regression analysis to see if UGT2B15 and UGT2B17, like ADRB2, were associated with development of CRPC in the Oslo ADT cohort. 33 and 34 out of the 45 patients had successful immunohistochemical staining of tumor tissue with UGT2B15 and UGT2B17, respectively. Weak UGT2B15 staining intensity was significantly associated with a more rapid development of CRPC (SHR 0.39, 95% CI 0.16-0.97, *p*-value 0.043), while UGT2B17 was not (Table 3). Neither UGT2B15 nor UGT2B17 were associated with prostate cancer specific or all-cause mortality (Table 3a and 3b, respectively).

DISCUSSION

We report that prostate cancer patients expressing low levels of the β_2-adrenergic receptor in the cancer tissue more rapidly develop castration-resistant prostate cancer. Furthermore, xenograft tumors from prostate cancer cells with knockdown of ADRB2 were shown to grow more rapidly in castrated mice than xenografts tumors from control cells. We have identified a novel mechanism by which ADRB2 may indirectly regulate the activity of the androgen receptor in prostate cancer cells, namely through regulating glucuronidation, a critical step in the extrahepatic phase II metabolic elimination pathway of androgens. LNCaP cells expressing low ADRB2 levels showed reduced UGT2B15 and UGT2B17 expression and activity compared to cells expressing high ADRB2 levels. The low ADRB2 expressing LNCaP cells were more responsive to androgen stimuli, displayed increased testosterone levels and a higher basal androgen receptor activity. Furthermore, supplementation with the competitive UGT2B-substrate diclofenac enhanced androgen responsiveness in high-ADRB2 expressing LNCaP cells, with a simultaneous increase in the intra-cellular testosterone level.

Like androgens, adrenergic stimulation contributes to prostatic differentiation *in vivo* [23]. Moreover, ADRB2 signaling activates androgen responsive promoters *in vitro* and is therefore suggested to play a role in development of CRPC [12]. Short-term activation of ADRB2 stimulates androgen receptor activity [12], while long-term activation of ADRB2 leads to desensitization of ADRB2 [24]. Furthermore, down-regulation of ADRB2 induces de-differentiation and epithelial to mesenchymal transition (EMT) [20], a process associated with CRPC development. This is the first study addressing whether ADRB2 correlates with CRPC *in vivo,* and the data are consistent with the hypothesis that ADRB2 is associated with CRPC.

This study points to a novel mechanism by which long-term knockdown of ADRB2 may support CRPC development. In xenografts, LNCaP tumors expressing low levels of ADRB2 have a shorter lag period and grow more rapidly after castration than tumors with normal ADRB2-levels, indicating that these cells may be more adapted to an androgen-deprived milieu. Being more adapted to castration theoretically predicts therapy failure or imminent recurring growth, which seems to be the case for these shADRB2 tumors.

The increased testosterone levels and enhanced androgen responsiveness observed in ADRB2 knockdown cells may relate to the observation that androgen-glucuronidating activity is down-regulated. Reducing glucuronidation could preserve residual and *de novo* biosynthesized androgens and thus rescue androgen receptor stimulation, which would give the cells an "edge" in an androgen-deprived micro milieu. Thus, this may represent an adaption mechanism by which the cells maintain a sufficient androgen receptor activity to uphold survival. *In vivo,* this mechanism may complement the well-known increase in intra-tumoral androgen biosynthesis and androgen receptor expression observed in CRPC [25-27].

The cAMP signaling pathway is an essential inducer of steroidogenesis in steroidogenic cells [14-17]. To what extent β-adrenergic signaling regulates steroid synthesis in prostate cancer cells is not known, but our study suggests that the receptor may be involved in regulating the amount of bioactive androgen through modulating glucuronidation activity. Testosterone levels were increased both in cells expressing low levels of UGT2B15 and UGT2B17, and in cells treated with diclofenac, which has previously been reported to be a UGT2B15 and UGT2B17 competitive inhibitor [28-30]. Furthermore, stimulation with the synthetic androgen R1881, reported to be non-glucuronidable [31], gave similar androgen responses in the shADRB2 (low UGT2B) and shCtrl (high UGT2B) cell lines, indicating that glucuronidation regulates the observed differences in androgen responsiveness solitarily. In support of this, a study by Chouinard et al., showed that knockdown of UGT2B15 and UGT2B17 in LNCaP cells lead to a more pronounced modulation of androgen-regulated genes [32].

Several studies have investigated the expression level of UGT2B15 and UGT2B17 in hormone naïve and castration resistant prostate cancer [9, 25, 33]. Collectively, none of these studies show a significant difference in immunohistochemical staining intensity for neither UGT2B15 nor UGT2B17 expression between androgen-dependent prostate cancer and CRPC. No study has yet, however, investigated how UGT2B15- or UGT2B17 expression in hormone naïve prostate cancer relates to time to development of CRPC. In our analyses, UGT2B15 staining intensity was statistically significantly correlated with CRPC development, while UGT2B17 was not. Both enzymes, however, are positively correlated with ADRB2 staining, which itself was associated with CRPC development. The positive correlations between the two UGT2Bs and ADRB2 in tissue samples support our observations of reduced UGT2B15 and UGT2B17 levels

after knockdown of ADRB2 in LNCaP cells. Furthermore, the finding that differences in glucuronidation activity between shCtrl and shADRB2 cells were maintained in our mouse model after castration, points to the possibility that ADRB2 may influence glucuronidation activity also in humans.

If UGT2B15 and UGT2B17 are important determinants of the availability of bioactive androgens in the tumor micro milieu *in vivo*, patients with low ADRB2 expression may have a lower response to androgen-deprivation therapy through lowered UGT2B15 and UGT2B17 protein levels. This might explain our observation that low ADRB2 levels are associated with a poor prognosis.

It should be noted that diclofenac is a non-steroidal anti-inflammatory drug, and may thus affect pathways that directly or indirectly affect androgen receptor activity, i.e. through affecting prostaglandin metabolites that are known to inhibit AR [34]. Whether this in turn could affect our reported effects on androgen responsiveness was not assessed in this study.

Low level of ADRB2 has previously been shown to predict a shorter time to clinical failure after radical prostatectomy, as defined by biochemical recurrence [20]. It is worth noting, however, that 60-70% of men experience recurrence without emergence of clinical symptoms, and only around 8% of patients that experience biochemical recurrence die from prostate cancer [35]. Thus, clinical progression probably serves as a better end point in biomarker studies. We used clinical progression as an end point, and see that ADRB2 may act as a prognostic biomarker for CRPC. Furthermore, alterations in androgen glucuronidation activity are presented as one potential mechanism by which ADRB2 may regulate development of castration resistant prostate cancer.

MATERIALS AND METHODS

Ethics

The Regional Ethical Committee (s-04153c), the Data Protection Official at Oslo University Hospital (41-2009 AUS) and The Norwegian Data Protection Authority (09/00450-2 /bso) has approved this study. Written consent was obtained from all surviving patients, and a permission to include clinical information on deceased patients was obtained from the Regional Committee for Medical and Health Research Ethics (2009/1028).

Patient material and TMA construction

For the Oslo ADT TMA, 61 patients treated with palliative transurethral resection of the prostate (TUR-P) and ADT at Oslo University Hospital, Aker, in the period 1992- 2008 were identified retrospectively from medical records. 16 patients were excluded from the analyses due to lack of sufficient tissue or lack of consent.

Clinical information was obtained from medical records. Retracted data included date of birth, date of diagnosis, date of initiation of hormonal treatment or orchiectomy, and date of progression. Date and cause of death were obtained from Statistics Norway, per May 1st 2012.

Tissue was obtained from “The Prostate Biobank- a resource for urological research in Norway” (No.119 The Biobank Registry at Norwegian Institute of Public Health). One area representing normal and two areas representing prostate cancer tissue were marked on hematoxylin/eosin stained sections, extracted using a 0.6 mm tissue core, and mounted using a semi-motorized tissue arrayer (TMABooster, Alphelys, Plaisir, France).

The treatment initiation date was set at the first time of administration of anti-androgen, luteinizing hormone- releasing hormone (LHRH) agonist, or date of orchiectomy, where applicable. Where the exact date of diagnosis, initiation of ADT or disease progression was not noted in the patient’s journal, the actuar-method was used to assign an event date; that is, the middle date between two known dates before and after diagnosis, start of hormonal treatment, or disease progression.

Patients were considered to have CRPC in the case of two consecutive PSA rises, progression to metastatic disease, or when noted explicitly in the patients journal.

Briefly, for the TMA constructed from specimens obtained from the “Vancouver Prostate Centre Tissue Bank” [36], H&E-stained slides were inspected and desired areas of 1 mm were extracted and mounted manually (Beecher Instruments, MD, USA) as duplicated cores. Among the 304 prostate cancer specimens, 143 were from radical prostatectomies, 96 were from radically operated patients who had been pretreated with androgen deprivation therapy for one to twelve months prior to surgery, and the remaining 23 were CRPC samples obtained through TUR-P. The patients were operated in the period 1999-2009.

Immunohistochemistry

For the Oslo ADT TMA, TMA sections of 4 μm were deparaffinized and antigens were retrieved at 97°C for 20 minutes using the PT-link (Dako, Glostrup, Denmark) and “Target Retrieval Solution, high pH” (K8004, Dako) for ADRB2 whereas the slide was microwaved for 10 min in citrate buffer (pH 6.0) (Thermo Fischer Scientific, Waltham, MA) and immersed in 0.5% v/v hydrogen peroxide/methanol for 20 min for UGT2B15 and UGT2B17 immunostaining. The anti-ADRB2 antibody (MC2656, MBL International, Woburn, MA), the anti-UGT2B15 antibody [37], and the anti-UGT2B17 antibody [38] were used in dilution 1:400, 1:500 and

1:500, respectively. The immunostainings were visualized using the Envision Flex" (K8010, Dako) kit. Images were captured using a Zeiss AXI0 Imager.A1 microscope with an attached Zeiss AxioCamERc5s camera (Zeiss, Oberkochen, Germany) using Histolab 8 (Alphelys, Plaisir, France), and manual scoring of the staining was performed by pathologists AS and WW (ADRB2) or AS and BK (UGT2B15 and UGT2B17). For survival analyses, staining intensity for the spot(s) with the highest apparent Gleason score was chosen for further analysis. Where the tissue in a spot showed more than one staining intensity, fractions were used. In the case of two spots with the same apparent Gleason score from one patient, or disagreement regarding staining intensity, the average intensity was calculated and used in the analyses. The Gleason scores were determined by two experienced uro-pathologists (AS and WW).

ADRB2 antibody specificity and sensitivity was tested, and is shown in Supplementary Material and Methods.

The immunohistochemical staining of TMAs in the "Vancouver Prostate Centre Tissue Bank" was performed as previously described [36]. Pathologist LF evaluated staining intensities, and the staining intensities in the dataset were exclusively used for correlation analyses.

For the immunohistochemical analyses of xenograft tumors, the paraffin-embedded tumors were in brief sectioned, mounted onto glass slides, and stained with UGT2B15 (1:500) or UGT2B17 (1:500) antibodies. Pathologist BK scored staining intensities.

Plasmids

Two short hairpin SureSilencing™ shRNA plasmids with insert sequences targeting ADRB2 mRNA, as well as a non-targeting shRNA as control, were purchased from Qiagen (Supplementary Table 1) (Qiagen, Hilden, Germany). Two androgen-responsive reporter constructs were used including the probasin and PSA promoters, respectively. The PSA reporter plasmid (pGL3/PSA) included 7 kb of the 5'- upstream region of PSA [39]. The probasin promoter sequence (pPB(-285/132)-LUC [40]) was cloned into a Gluc-ON™ Promoter clone system (pEZX-PG04, GeneCopoeia) expressing Luc and Secreted alkaline phosphatase (SEAP) as tracking gene (pEZX-PG04-Pb-LUC). An empty pEZX-PG04 vector expressing SEAP was used to control for differences in transfection efficiency. A pCDNA3.1 vector, expressing the ADRB2 gene (pCDNA3.1-ADRB2), was used for over-expression [41].

Cell lines

LNCaP cells (ATCC (VA, USA), purchased 10/2009), were maintained in RPMI 1640 containing 10% Fetal Bovine Serum (FBS) ((Sigma-Aldrich, St. Louis, MO), 100units/ml penicillin and 50mg/ml streptomycin (InVitrogen, Carlsbad, CA) at 37°C with 5% CO_2 and humidified air, and were given fresh medium every 48 hours. Stable ADRB2 knockdown was achieved by transfection of LNCaP cells at passage 25 with three different shRNA sequences (two different ADRB2 shRNAs; shADRB2-1 and 2, and a non-targeting shRNA; shCtrl), using Dharmafect Duo (Dharmacon/GE). Stably transfected cells were maintained in 200µg/ml G418 sulphate. Cells were exclusively used between passage 28 and 45. Cell IDs of parental LNCaP, LNCaP shADRB2 and shCtrl cell lines were verified using the STR PowerPlex16 System (Promega, Fitchburg, WI) (tested 07/2014).

Stimulation of cell cultures

To study effects of androgen stimulation, LNCaP cells were grown in phenol red free RMPI 1640 (Life technologies, Carlsbad, CA) added 2% charcoal-stripped FBS (CSS, Gibco, Carlsbad, CA) and supplemented with either 1.0 or 10 nM metribolone (R1881, Roussel UCLAF), 1.0 or 10nM dihydrotestosterone (DHT, kindly provided by the Hormone Laboratory, Oslo University Hospital), 50 µM diclofenac sodium (DFS, Cayman Chemical Company, MI) or vehicle (ethanol). The cells were preincubated in RPMI with 2% CSS where noted.

Animal experiments

Twenty-one in-house bred, 4 week old male NOD-SCID gamma/null mice weighing 28.2 ± 4 g were administered 0.03mg/ml testosterone (Sigma-Aldrich) in drinking water one week prior to s.c. injection into the hind flank with 2 x 10^6 LNCaP shADRB2-2 (n = 10) or shCtrl (n = 11) cells suspended in Matrigel (1:1) (BD Biosciences, San Jose, CA). Tumor volumes were assessed weekly using caliper measurements and calculated by the formula: (length x width^2)/2. Once tumor size reached 500mm^3, the mice were surgically castrated under anesthesia by removal of testes and taken off testosterone supplementation. The mice were sacrificed when the tumor volumes reached 2000 mm^3. The tumors were excised and split in two parts: One part was fresh frozen and used for measurement of glucuronidation activities, the other formalin-fixed and paraffin-embedded for immunohistochemical analyses. The experiment was approved by the National Animal Research Authority (FOTS ref. 7132) and was performed according to regulations of the Federation of European Laboratory Animals Science Association.

Transient transfection and reporter assays

The LNCaP sub-cell lines were transfected using the Dharmafect Duo reagent. Reporter activities (secreted luciferase and SEAP) in medium samples were measured 48 hours after transfection using the Secret-Pair Dual Luminescence Assay kit (GeneCopoeia) on a Victor Wallac Spectrophotometer (PerkinElmer, Waltham, MA). To determine intra-cellular luciferase activity cells, cells were lysed in 1X Reporter Lysis buffer (Promega), the supernatant mixed with Luciferase Assay Reagent (Promega) and the activity measured on a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

RNA extraction and real-time RT-PCR

Total RNA was extracted using the TRIzol reagent following manufacturers protocol (Invitrogen). 100 ng of total RNA was used in the qScript™ One-Step qRT-PCR Kit (Quanta Biosciences, Gaithersburg, MD). The RT-PCR reactions were performed on a CFX Connect™ Real-Time System (BioRad, Hercules, CA) under 48° C for 10 min; 95°C for 5 min for the first cycle; 95°C for 15 s, 55°C for 30 s for 40 cycles; 60 melt curve read offs from 65-95°C. ALAS-1 or POLR-2A mRNA expression were used for reference. To display relative gene expression, the $\Delta\Delta C_t$ formula [42] was used. The primers used were: ADRB2 fwd: gtcttgagggctttgtgctc, rev: ggcagctccagaagattgac; UGT2B15 fwd: gatcatcgaccccagagaaa, rev: tcactgtaaaccagccaaacc; UGT2B17 fwd: gatcatcgaccccagagaaa, rev: cgcccattcttaccaaatgt; Kallikrein 3/Prostate Specific Antigen (KLK3/PSA) fwd: ccctgagcacccctatcaac, rev: tgagtgtcggtgggttgtg.

Prostate specific antigen (PSA/KLK3)

Total PSA in medium was determined by the AutoDELFIA ProStatus PSA Free/Total Kit (PerkinElmer Inc., USA) by time-resolved fluorescence on the AutoDELFIA instrument. Total PSA was normalized to the amount of protein in each sample.

Protein extraction and immunoblot analysis

The cells were harvested, lysed in whole cell buffer [43], and centrifuged at 16,000 g for 20 min. Immuno-blots were prepared and visualized as previously reported [37, 38]. Anti-UGT2B15 (1:1500) or anti-UGT2B17 (1:2000) was used as primary antibodies, and anti-actin (1:2000, #A5060, Sigma-Aldrich) as loading control. Both UGT2B15 and UGT2B17 antibodies were kindly provided by A. Bélanger (CHU-Québec research centre) [38].

Radioligand binding and adenylyl cyclase assays

Cell membrane fractions were prepared as described in [44], and the ADRB2 protein binding activity was measured by radioligand binding assay, as previously described [45] with a binding buffer described in [46]. The total number of specific binding sites was determined. Ligand binding was normalized to the total amount of protein in the membrane fractions.

Adenylyl cyclase activity was measured by determining conversion of [α-^{32}P]ATP to [^{32}P]cAMP in cell membrane fractions in the presence and absence of 10 µM isoproterenol (Sigma-Aldrich) for 20 minutes, and was related to whole membrane protein as previously described [44].

Formation of steroid glucuronides

LNCaP shADRB2 and shCtrl protein lysates were prepared by centrifugation at 890g for 10 min at 4°C followed by lysis by sonication on ice. The lysates were diluted in PBS supplied with 0.5 mM dithiothreitol (DTT) (GE Healthcare, Buckinghamshire, UK). Xenograft homogenates were prepared by homogenizing 50 µg of tumor tissues from mice injected with LNCaP shADRB2 and shCtrl in 250 µL of ice-cold PBS supplied with 0.5 mM DTT using a Homogenizer Motor Drives (Glass-Col Homogenizer #099C-K54, Terre-Haute, IN, USA). Steroid glucuronidation assays were performed by adding 10 µL of lysates (8.8 µg/µL) or xenograft homogenates (5 µg/µL) to a glucuronidation assay buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl2, 10µg/mL phosphatidylcholine, 1 mM uridine 5'-diphosphoglucuronic acid (UDPGA), 2.5 µg/mL pepstatin, 0.5 µg/mL leupeptin, 0.025 µg/mL alamethicin, and 100 µM of either testosterone, DHT, 3α-Diol, or androsterone dissolved in ethanol). The mixtures were incubated at 37°C for 1 or 4 hours (cell lysates or xenograft homogenates, respectively) before quenching the glucuronidation reactions with 2 nM ice-cold methanol:butylated hydroxytoluene (BHT). Proteins were centrifuged at 13,000 rpm for 10 minutes at 4°C to pellet the protein precipitate. Supernatants were used for glucuronide quantification by LC-MS/MS as previously reported [47]. Cell lysate samples used for glucuronidation assays were prepared with the same dilutions of those used for immunoblotting assays, so the protein levels directly correspond to the glucuronidation activity. HEK293-cells and ethanol were used as negative controls, and a pool of human liver samples as positive control.

Androgen quantification

Testosterone levels in cell cultures was measured by a multi-steroid LC-MS/MS assay as described by [48]

with the following modifications: Isotope-labeled internal standards (10 µl) and 190 µl 50 % acetonitrile was added to 85 µl of each calibrator and quality control as well as to each cell pellet lysate. The samples were sonicated (40% amplitude, 10 pulses of 1s), equilibrated at RT for one hour, and extracted by liquid-liquid extraction with 850 µl ethylacetate:hexane (80:20). After centrifugation, 650 µl of the organic phase was dried under a stream of N_2 for 30 minutes at 40°C. The samples were reconstituted in 50 µl 25% methanol, and 10 µl of each sample was injected into a Waters Xevo TQ-S that was coupled to an i-class Acquity UPLC. The chromatographic system consisted of a 100 x 2.1 mm Acquity BEH C-18 column (1.8 µM particle size) heated to 60° C, and two mobile phases consisting of Milli-Q purified water with 0.05% ammonium hydroxide (A) and methanol with 0.05% ammonium hydroxide (B). Samples were separated by a linear gradient from 30% to 75% mobile phase B over 7.5 min at a flow-rate of 0.4 ml/min. Testosterone was quantitated by electrospray ionisation in positive mode, and multiple reaction monitoring as described previously [48]. Concentrations were calculated as fmol per cell pellet or tumor tissue weight.

Statistics

Time to development of CRPC and time to prostate cancer-specific mortality was analyzed by competing risk regression using the Fine and Grey model [49], while Cox proportional hazards modelling was applied for all-cause mortality analysis. The proportional hazards assumption was assessed by a test based on Schoenfeld residuals. There was no evidence of violation of the assumption in any model (all *p*-values > 0.13). Correlations were calculated using Spearman's rank correlation, due to non-normality of the data. For all other statistical analyses, a two-tailed Student's t-test or Fischer exact test were used to determine statistical significance. For xenograft tumor glucuronide formation, values were $\log_{10}$-transformed to invoke normally distributed data prior to parametric testing. Statistics were performed using SPSS version 18, STATA version 12 and Microsoft Excel. A two-sided *p*-value of < 0.05 was considered statistically significant for all analyses.

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CONFLICTS OF INTEREST

The authors disclose no potential conflicts of interest.

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Low β_2-adrenergic receptor level may promote development of castration resistant prostate cancer and altered steroid metabolism

Supplementary Information

MATERIAL AND METHODS

ADRB2 antibody specificity and sensitivity

Multiple antibodies targeting ADRB2 were tested on western blots, and the antibody chosen for the immunohistochemical staining recognized both the correct band from an ADRB2 over-expression lysate from HEK293T-cells (LY424968, Origene, Rockville, MD) and only one single band from patient tissue protein extracts which' size corresponds to the theoretical size. The antibody only bound luminal cells, which is in compliance with prior immunohistochemical analyses using other antibodies in prostate tissue. Finally, immunofluoresence of shADRB2 cells showed reduced signal intensity (data not shown).

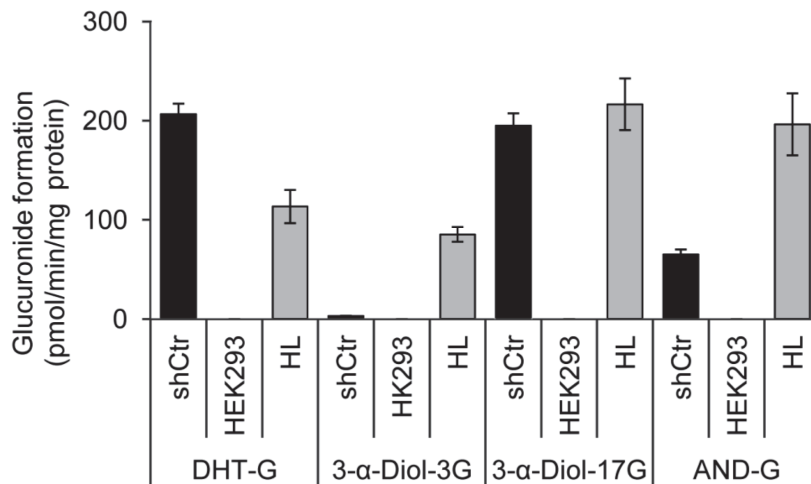
Supplementary Table 1: List of plasmid names, vectors, comments and the source/reference of the plasmids.

Plasmid name	Vector	Comment/insert	Source/reference
shCtrl	SureSilencing shRNA plasmid	GGAATCTCATTCGATGCATAC	Qiagen (Cat no. KH01856N)
shADRB2-1		TGAGACCTGCTGTGACTTCTT	Qiagen (Cat no. KH01856N)
shADRB2-2		GGCAACTTCTGGTGCGAGTTT	Qiagen (Cat no. KH01856N)
pCDNA3.1-ADRB2	pCDNA3.1	Constitutively expresses ADRB2 with a Flag tag protein	Tang et al. 1999
pEGFP-C3	pEGFP-C3	Constitutively expresses GFP under CMV-promoter	Clontech
pPB(-285/132)-Luc	pGL3	Gift from F. Saatcioglu (University of Oslo, Norway)	Palvimo et al. 1996
285-Pb-pEZX-PG04	pEZX-PG04	pPb(-285/132)-Luc was Cloned into Gluc-ON™ Promoter clone system	GeneCopoeia
pEZX-PG04	pEZX-PG04		GeneCopoeia
pGL3/PSA	pGL3	Gift from S. Balk (Dana-Farber/Harvard Cancer Center, MA)	Oettgen et al. 2000

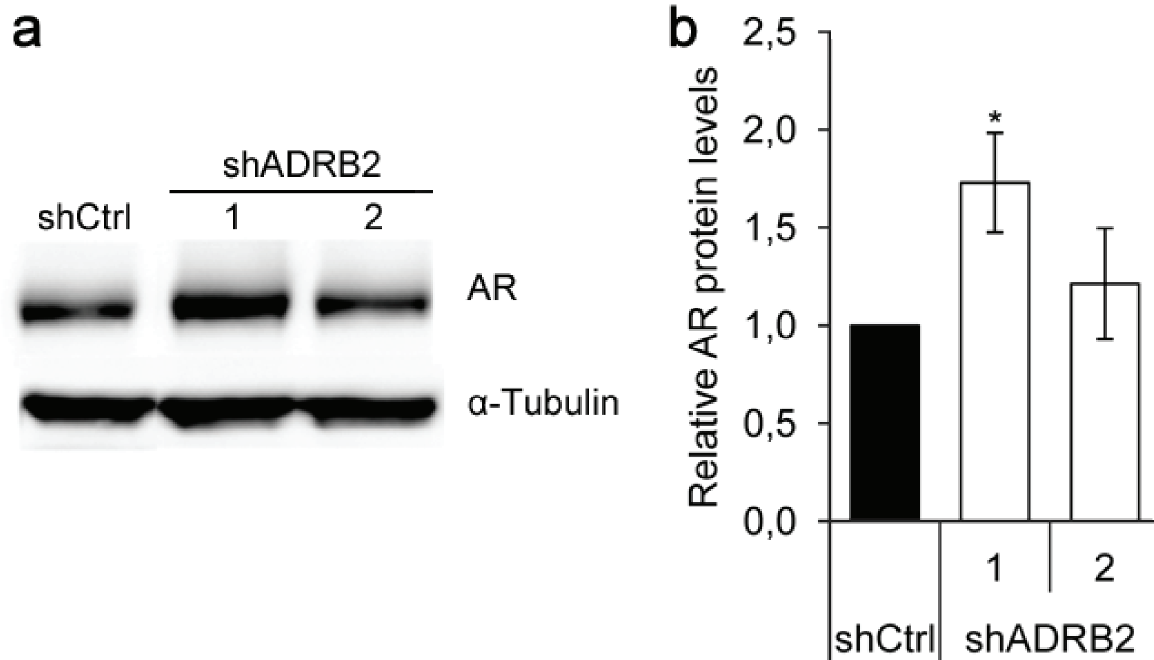
Androgen receptor (AR) western blots

The LNCaP shADRB2 and shCtrl cells were harvested, lysed in whole cell buffer, and centrifuged at 16,000 g for 20 min. Immuno-blots were prepared and visualized as described in materials and methods. Anti-AR (1:1000, Santa Cruz Biotechnology, TX, USA) was used as primary antibody, with α-tubulin (1:1000, Sigma-Aldrich) as loading control.

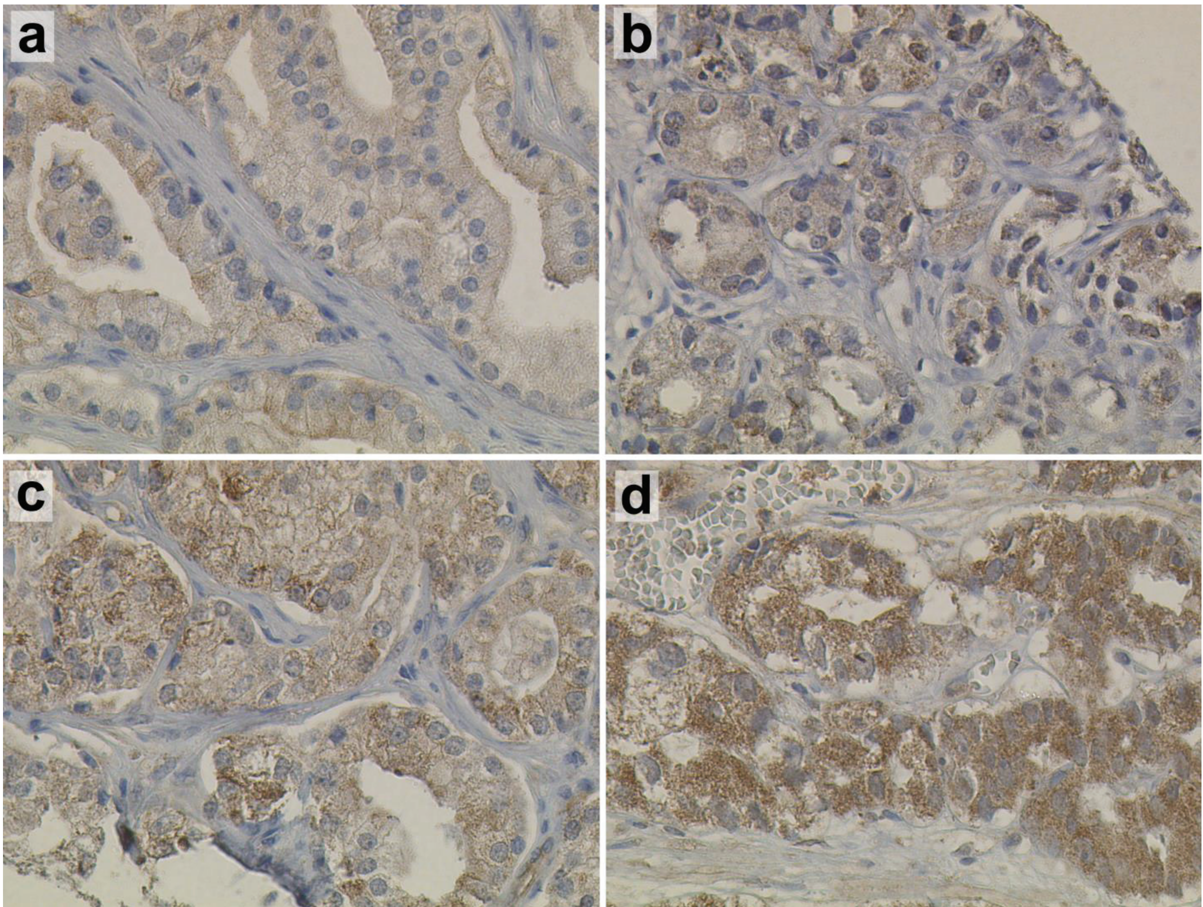
RESULTS



Supplementary Figure 1: Formation of androgen glucuronides in positive and negative controls. Homogenates from HEK293 cells (negative control) and human liver samples (HL, positive control) were mixed with UDPGA and either dihydrotestosterone (DHT), 3α-androstanediol (3α-Diol) or androsterone (ADT) for one hour, and levels of conjugated androgens were measured by LC-MS/MS. The results are shown as mean formed glucuronide related to total protein in the homogenates (pmol/min/mg protein) from duplicated reactions on three biological replications ± SEM. The values obtained with LNCaP shCtrl are shown to compare glucuronidation rates with the controls.



Supplementary Figure 2: Androgen receptor (AR) protein level. (a) The AR protein level was visualized in cell protein extracts by immunoblotting using an anti-AR antibody (1:1000). An anti-tubulin antibody (1:1000) was simultaneously used on the same extracts to ensure similar loading on the lanes. **(b)** Mean, relative AR protein intensities from three independent western blot experiments are shown ± SD.



Supplementary Figure 3: Immunohistochemical analysis of UGT2B15 and UGT2B17 expression in the Oslo ADT TMA of transurethral resections of the prostate (TUR-P). Examples of tissue cores of Gleason 7b showing **(a)** weak UGT2B15, **(b)** intermediate UGT2B15, **(c)** weak UGT2B17**,** or **(d)** intermediate UGT2B17 staining are presented at 400X magnification.

Supplementary Table 2: Clinical characteristics at diagnosis for patients included in the immunohistochemical analyses of ADRB2.

Clinical characteristic at diagnosis	Number of patients (n=40)
Gleason score	
2-6 or highly differentiated	13
7 or intermediately differentiated	13
8-10 or poorly differentiated	14
Unknown	0
Clinical T-stage	
1-2	5
3-4	14
Unknown	21
PSA-level	
< 4.0	0
4.1-10.0	3
10.1-20.0	5
> 20.0	19
Unknown	13
Metastasis	
Yes	3
No	20
Unknown	17
Age at diagnosis (mean (SD))	69.3 (8.3)

Errata

Name of candidate: Peder Rustøen Braadland

Thesis title: Targeting Therapy Resistance in Advanced Prostate Cancer

Abbreviations: Cor = Correction, Ital = Italicization, Rep = repetition

Page	Line #	Original text	Corrected text	Type
15	15	...to develop (Figure 3.1). Finally, by identifying...	...from developing (Figure 3.1). Finally, identifying…	Cor
20	8	... which are exploited...	...which is exploited...	Cor
21, 29, 52	3, 7, 13	KLK3, AR, ADRB2	*KLK3, AR, ADRB2*	Ital
23	4	...modality over other...	...modality over the other...	Cor
23	11	...after RP, but these...	...after RP, these...	Cor
23	29	...(Eulexin) bicalutamide (Casodex) offered...	...(Eulexin), bicalutamide (Casodex) are offered...	Cor
24	19	...and is achieved by...	...and is accomplished by...	Rep
25	16	...yet assessed...	...yet been assessed...	Cor
30	25	...enzalutamide also exist...	...enzalutamide exist...	Cor
31	17	...bicalutmide resistance than...	...bicalutamide-resistant than...	Cor
32	26	...the phosphoinositide 3 kinase/protein kinase B (PI3K/AKT)...	...the PI3K/AKT...	Rep
32	32	...The co-localization of AR...	...Co-localization of the AR...	Cor
33	23	...allows the cell...	...allows a cell...	Cor
34	3	...indications benefiting from...	...indications for...	Cor
37	4	...plasticity are clear...	...plasticity are unclear...	Cor
39	18	...H3K27me3 have...	...H3K27me3 levels have...	Cor
39	24	...showed illustrated...	...showed...	Rep
44	5	..with AR expression score...	...with an AR expression score...	Cor
47	16	...through regulation epithelial...	...through regulating epithelial...	Cor
49	3	...associated...	...associates...	Cor
49	19	...by 55% in...	...by 55%, respectively, in...	Cor
51	22	1998	1988	Cor
59	23	... Gleason score (GS)...	...Gleason score...	Cor
69	18-19	...analyses at the University of Finland, which also revealed a...	...analyses revealing a...	Rep
75	34	...reactivation the AR...	...reactivation of the AR...	Cor
78	14	...Wnt/beta-catenin...	...Wnt/β-catenin...	Cor
79	12	...depletion occurs in...	...depletion takes place in...	Rep
79	24	...*et al*., N/NC stem-transition...	...*et al*., switching to a N/NC stem-transition...	Cor
81	20	...with NE/t-NEPC,...	...with NED/t-NEPC,...	Cor
85	26	...groups who will...	...groups that will...	Cor