2020

Characterizing glucocorticoid receptor in metastatic castration resistant prostate cancer

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

CHARACTERIZING GLUCOCORTICOID RECEPTOR IN METASTATIC CASTRATION RESISTANT PROSTATE CANCER

by

MATTHEW ADAM KAHN

B.S., Syracuse University, 2018

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2020

Approved by

First Reader _____

Linda M. Afifi, Ph.D.
Assistant Professor of Anatomy & Neurobiology

Second Reader _____

Sujit S. Nair, Ph.D.
Assistant Professor of Urology
Icahn School of Medicine at Mount Sinai

ACKNOWLEDGMENTS

I would like to thank my supervisors Dr. Dimple Chakravarty and Dr. Sujit Nair for all of their support, dedication, and guidance from the moment I began working at Mount Sinai. Furthermore, I would like to thank Dr. Linda Afifi for her unwavering help and eagerness to assist me with this thesis project.

CHARACTERIZING GLUCOCORTICOID RECEPTOR IN METASTATIC CASTRATION RESISTANT PROSTATE CANCER

MATTHEW ADAM KAHN

ABSTRACT

The purpose of this paper is to characterize the glucocorticoid receptor (GR) signaling and relevance in the context of enzalutamide resistant prostate cancer cells. Enzalutamide is a drug that functions to dampen androgen receptor (AR) signaling, thus inhibiting cancer dependency on the receptor protein. Although the application of the drug reduces AR signaling in these cancer cells, an alternate pathway involving GR signaling may be upregulated as a compensatory bypass mechanism. Therefore, it possible that GR assumes the role of AR and facilitates tumor growth by promoting the expression of genes regulated by AR. To analyze how GR operates, we analyzed GR signaling in enzalutamide resistant metastatic prostate cancer cell lines. We assessed protein levels of AR and GR as well as mRNA expression of various AR targets. Our results illustrate the expected downregulation of AR and upregulation of GR in enzalutamide resistant cells. Furthermore, some canonical AR targets like prostate specific antigen (PSA), Prostate Specific Membrane Antigen (PSMA) and Prostatic Acid Phosphatase (PAP) were inhibited by a novel GR inhibitor. Thus, this GR inhibitor could be used in combination with enzalutamide and create a more potent AR signaling blockade. Prostate cancer is a very problematic disease in men and becomes especially challenging to treat during the metastatic stage as they are non-sensitive to anti-androgens. The significance of understanding how GR functions, as well as the potential

benefit of blocking GR signaling, may provide insight into novel drugs and agents that could specifically target these pathways, control and mitigate cancer growth, and prolong the lives of patients.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

aBSI..... Automated Bone Scan Index

ADT ..... Androgen Deprivation Therapy

AR ..... Androgen Receptor

ARE..... Androgen Response Elements

BM-PCa ..... Bone Metastatic Prostate Cancer

CRPC .....Castrate Resistant Prostate Cancer

CSPC.....Castrate Sensitive Prostate Cancer

DBD ..... DNA Binding Domain

DHT ..... Dihydrotestosterone

FSH .....Follicle Stimulating Hormone

GH..... Growth Hormone

GnRH .....Gonadotropin-releasing Hormone

GR ..... Glucocorticoid Receptor

IGF-1..... Insulin-like Growth Factor-1

IGFBP-3..... Insulin-like Growth Factor Binding Protein 3

LH .....Luteinizing Hormone

LH-RH ..... Luteinizing Hormone-releasing Hormone

LNCaP..... Lymph Node Carcinoma of The Prostate

LND ..... Lymph Nodal Dissection

LNI.....Lymph Node Invasion

LREX .....LNCaP/AR Resistant to Enzalutamide Xenograft

MRI ..... Magnetic Resonance Imaging

OS ..... Overall Survival

PAP ..... Prostatic Acid Phosphatase

PET ..... Positron Emission Tomography

PFS ..... Progression-free Survival

PLND ..... Pelvic Lymph Node Dissection

PSA ..... Prostate Specific Antigen

PSMA ..... Prostate Specific Membrane Antigen

qPCR ..... Quantitative Polymerase Chain Reaction

SPECT/CT ..... Single Photon Emission Tomography with Computed Tomography

TBU ..... Total Bone Uptake

VCaP ..... Vertebral Cancer of the Prostate

INTRODUCTION

1.1 Overview

Prostate cancer is one of the leading diseases in men around the world and is the second most diagnosed cancer in this group (Bray et al., 2018). Studies outlining the comparative incidence rates in United States throughout several years has shown that in 2004 there was an incidence of 68,814 cases as opposed to 2013 in which there were 67,070 cases. From 2004 to 2013 there was a total of approximately 767,550 diagnosed cases. Given that prostate cancer has different stages of development and thus varying levels of risk at the point of diagnosis, stratification of low risk, intermediate risk, high risk, and metastatic groups were used to assess incidence rates from years 2004 to 2013. There was a significant decrease in incidence of prostate cancer from 2007-2013 in the low risk group (30,323 to 16,223). There was a significant increase in incidence of prostate cancer from 2004-2008 (27,347 to 40,201) (Mohler et al., 2016). There were no significant changes from 2004 to 2013 for the high-risk group, but in terms of the metastatic group, there was a significant increase in the incidence from 2007 to 2013 (1884 to 2890) (Mohler et al., 2016). Significant decreases in incidence rates are most probably associated with a decline in the use of PSA testing as a method of early diagnosis. Skepticism over the accuracy of the biomarker as an indicator of early cancer has led medical professionals to research novel ways for detecting early stage prostate cancer (Siegel, Miller, & Jemal, 2019). The estimated number of new prostate cancer cases in 2020 in the United States is about 191, 930. The estimated number of deaths

associated with prostate cancer for the same year is about 33,330 (Siegel, Miller, & Jemal, 2020).

Prostate cancer, like many other diseases are associated with various factors that may contribute to the incidence or development of new cases. The more important factors in this case include age, race, genetic predisposition, and even environmental factors. Recent data from 2014 to 2016 has determined the probability of developing prostate cancer at different age groups (Siegel et al., 2020). In the United States, these include from birth to 49 years of age (0.2% or 1 in 441), 50 to 59 (1.8% or 1 in 57), 60 to 69 (4.7% or 1 in 21), and greater than or equal to 70 years (8.2% or 1 in 12). Most astonishingly, the death rate for prostate cancer has decreased by 52% since 1993 (Siegel et al., 2020).

From a world perspective, prostate cancer incidence rates are highest in regions of high human development like North America and Northern Europe. However, it is interesting to note that high mortality rates exist in countries with high proportions of African ancestry. These include the Caribbean countries of Barbados and Trinidad (Center et al., 2012; Warner et al., 2018).

1.2 Prostate Specific Antigen and Gleason Score

Historically, the emergence of prostate cancer in a patient and its course of development were indicated by the measurement of blood PSA levels. The seminal research conducted by Stamey *et al*. in 1987 discovered that the concentration of PSA in the blood for patients who did not undergo any prior treatment was indicative of the clinical stage of prostate cancer. Moreover, using multivariate regression analysis, it was

found that serum PSA concentration was proportional to the volume of the prostate cancer tumor. This was an important and vital step for the management of prostate cancer as it paved the way to gauge cancer burden on the patient and allowed for a potentially directive course for treatment (Stamey et al., 1987). However, there is not a definite threshold at which PSA serum concentration represents the presence of cancer. The standard protocol was to investigate the presence of cancer in the event that serum PSA was >4.0 ng/mL. A PSA concentration of <4.0 ng/mL was considered normal, however above this range, a urologist may recommend a patient biopsy (Thompson et al., 2004). Recent research has suggested that PSA as a biomarker for cancer is questionable due to the fact that high levels may be present in a patient with a clear absence of cancer (Azab, Osama, & Rafaat, 2012). Elevated total and free PSA levels could be related to other issues unrelated to cancer including prostatitis, urinary tract infections, and even masturbation (Azab et al., 2012; Dalton, 1989; Tarhan, Demir, Orcun, & Madenci, 2016).

PSA, also known as prostate specific antigen or kallikrein-3, is an enzyme that is a part of the kallikrein protein family (Yousef & Diamandis, 2001). The biomolecule initially presents as a preproPSA after translation. Subsequent cleavage steps yield the active 33 kDa PSA enzyme (Kumar, Mikolajczyk, Goel, Millar, & Saedi, 1997; Lundwall & Lilja, 1987). The regulation of the transcription of the PSA gene has been shown to be linked to the androgen receptor gene. Therefore, it is recognized as an androgen-regulated serine protease. The co-regulation of these transcriptional elements has important implications in the understanding of cancer growth and progression (Riegman, Vlietstra, van der Korput, Brinkmann, & Trapman, 1991). Typically, PSA presents in the seminal

fluid at a concentration of about 0.5 to 2.0 mg/mL (Lovgren, Valtonen-Andre, Marsal, Lilja, & Lundwall, 1999). Its function is to help liquify the semen by cleaving the gel-like semenogelins in the seminal coagulum. This effectively enables the sperm to swim freely (de Lamirande, 2007). Given that PSA is produced from the epithelial cells of the prostate gland and functions to cleave other proteins, it may have implications in the development and progression of prostate cancer (Catalona, Smith, Ratliff, & Basler, 1993; Pezaro, Woo, & Davis, 2014). Some research has shown that it has tumor-promoting qualities by cleaving insulin like growth factor binding protein 3 (IGFBP-3). IGFBP-3 binds to insulin-like growth factor-1 (IGF-1) which along with other molecules like growth-hormone (GH), help to facilitate somatic growth (P. Cohen, Peehl, Graves, & Rosenfeld, 1994).

Nevertheless, the importance of PSA can also be represented by the fact that higher PSA levels have been shown to correlate to higher tumor grades based on histopathologic findings from organ-confined prostate cancer. This suggests the association between PSA concentration and Gleason scores (Spencer, Chng, Hudson, Boon, & Whelan, 1998). Another groundbreaking work was conducted by Gleason *et al.* to characterize prostate cancer based on a standard grading and prognostic scoring system. Cancer scores of lower grades have healthier and normal looking tissue compared to higher grades which typically consist of abnormal tissue morphology (Gleason & Mellinger, 1974).

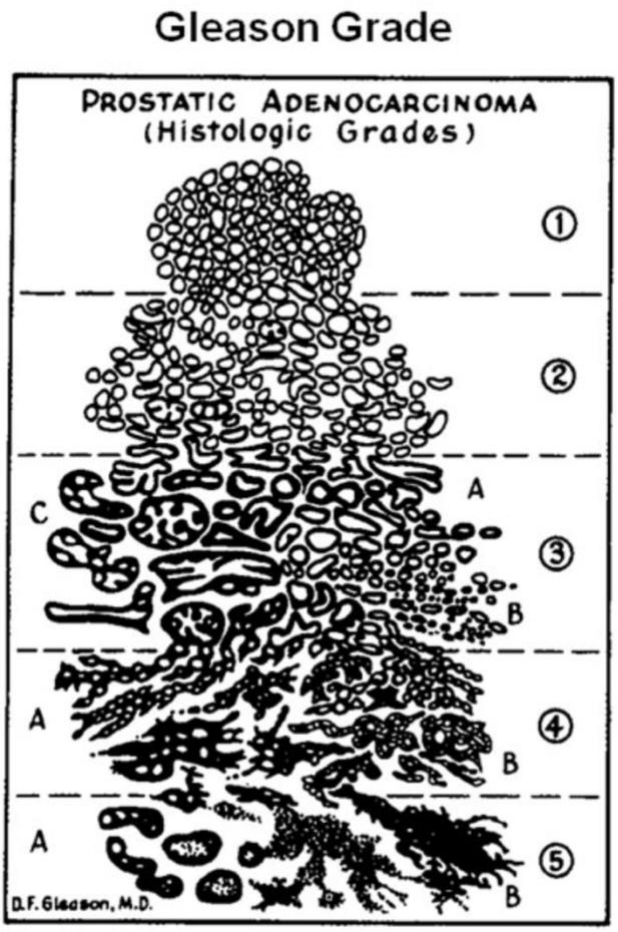


Figure 1: **Gleason's Pattern.** The Gleason grading system represents the progression from well differentiated to poorly differentiated/anaplastic prostate tissue (Gleason & Mellinger, 1974).

1.3 Progression of Prostate Cancer

Prostate cancer progresses through several phases including organ confined cancer growth, spread to local tissue like the seminal vesicles and other organs such as the neck of the bladder as well as the rectum, and finally metastasis to regional lymph nodes (Simmons, Berglund, & Jones, 2011). Metastasis can also occur in the blood spreading to distant areas of the body. The uncontrolled spread of metastatic prostate

cancer to the vertebral bone as well as the lungs becomes a very difficult state to manage and usually results in rapid death (Simmons et al., 2011).

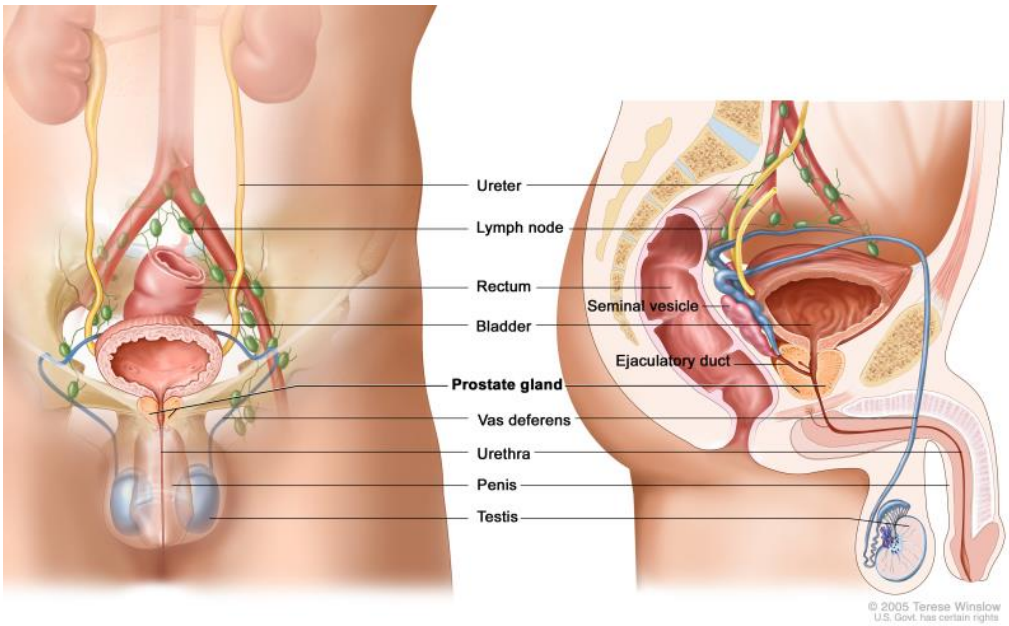


Figure 2: **Anatomy of the Male Reproductive and Urinary Systems.** (Adapted from PDQ Adult Treatment Editorial Board)

If prostate cancer is detected and diagnosed early, it presents as organ confined cancer, or growth directly from the prostate gland. This stage of prostate cancer is denoted as T2 using the TNM system (Table 1) for classifying each level of the prostate cancer. T2 can then be subdivided into several classes, T2a which is a unilateral lesion occupying less than 50% of affected side of the gland, T2b which is a unilateral lesion occupying greater than 50% of the affected side of the gland, and T2c which is a bilateral lesion (Billis et al., 2019). The urinary system consists of seminal vesicles that anatomically lie in close proximity to the prostate gland and therefore may be affected by prostate cancer spread. Seminal vesicles store and produce a lot of the fluid that makes up semen. The cancer spread to this region is therefore being studied intensively and

represents the T3 stage (Panach-Navarrete, Garcia-Morata, Hernandez-Medina, & Martinez-Jabaloyas, 2015). The T4 stage for prostate cancer signifies invasion to other organs such as the neck of the bladder as well as the rectum wall. Given that these organs have their own classes of cancers, a patient that has prostate cancer but presents with symptoms related something other than the prostate can be dangerous in the case of an incorrect diagnosis (R. J. Cohen, Li, & Shannon, 2016; Tang et al., 2017).

Prostate cancer can progress to more advanced stages in which there is lymph node involvement. Such a spread presents a very serious issue and a very dangerous problem for the patient because the lymph node if overwhelmed would compromise its immune function. Several areas of study have invested the impact of lymph node invasion (LNI) and prognostic factors associated with it in the advent of a patient presenting with prostate cancer. Typically, a method used to understand the extent of the invasion is the pelvic lymph node dissection (PLND). Patients that had PLND typically had more clinically aggressive symptoms and had a lower overall survival (OS) compared to patients that did not have PLND (Chen et al., 2019). For high-risk prostate cancer, PSA was a clinical factor associated with lymph node invasion. For example, median PSA values for extensive lymph node invasion was 23.5 ng/ml compared to 11.4 ng/ml for limited invasion and 7.3 ng/ml for no invasion ($p < 0.0001$) (Porcaro et al., 2018). Due to the invasiveness of such tests, usually as they are performed in conjunction with radical prostatectomy, the need for clear and harmless imaging techniques is paramount. Studies involving the PET scan uses a radioactive tracer to receive comprehensive structural information about the course of a diseased region. Currently,

choline and PSMA radiotracers are considered very useful in the context of lymph nodal dissection (LND) (Incerti, Mapelli, Gianolli, & Picchio, 2017). Furthermore, the minimally invasive MRI-guided pelvic lymph node biopsy has shown promise as a method for understanding suspicious nodal spread from prostate cancer (Hague et al., 2020).

The final and most lethal stage of prostate cancer is metastasis in the blood to distant sites like the bone and lungs. Treatment for metastatic prostate cancer is often very minimal due to the diffuse nature of the cancer throughout the body. Understanding the use of modern technology and imaging techniques can assist in gauging the cancer burden on the body and the specific diseased areas (Umeda et al., 2018). There are methods conducted to improve the detection of metastasis including the use of bone single photon emission tomography with computed tomography (SPECT/CT). Effectively, this method involves the calculation of total bone uptake (TBU), a measure of bone metastatic tumor burden (Umeda et al., 2018). Other studies have reviewed the use of automated Bone Scan Index (aBSI). It is an imaging tool that has implications in understanding survival in metastatic castration resistant prostate cancer. They used a multivariable model to understand the association between aBSI and overall survival by association with prognostic factors such as serum bone biomarkers (Armstrong et al., 2018). The first line of therapy post radical prostatectomy when there is a surge in PSA, is androgen deprivation therapy. However, the response to this treatment is mostly short lived and patients eventually develop resistance to the drug. This leads to aggressive tumor growth and metastatic spread of lesions. This is an active area of research and

several labs are invested in understanding resistance mechanisms and to identify drugs that can improve the response and contain tumor growth (Harris, Mostaghel, Nelson, & Montgomery, 2009; Scher & Sawyers, 2005). New techniques like immunotherapy may play a huge role in mitigating the effects of metastatic prostate cancer by stimulating the immune system for targeted cancer destruction. For example, studies on bone metastatic prostate cancer (BM-PCa) has elucidated the significance of neutrophils and their infiltration to these regions. Neutrophils are shown to be actively involved in the destruction of prostate cancer cells by directly inducing their apoptosis. However, in late stage BM-PCa, tumors are able to evade the cytotoxic effects of the neutrophils (Costanzo-Garvey et al., 2020). Metastasis can also occur in the lungs (Zang et al., 2015).

Table 1: **TNM Staging of Prostate Cancer.** (Cosma et al., 2016)

	Primary tumor (pT)
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
	Clinically inapparent tumor neither palpable nor visible by imaging (T1)
T1a	Tumor incidental histologic finding in $\leq$ 5% of tissue resected
T1b	Tumor incidental histologic finding in > 5% of tissue resected
T1c	Tumor identified by needle biopsy (e.g. because of elevated PSA)
	Tumor confined within prostate (T2)
T2a	Tumor involves one-half of one lobe or less
T2b	Tumor involves more than one-half of one lobe but not both lobes
T2c	Tumor involves both lobes
	Tumor extends through the prostate capsule (T3)
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
	Regional lymph nodes (pN)
NX	Regional lymph nodes were not assessed
N0	No regional lymph node metastasis
N1	Metastasis in regional lymph node(s)
	Distant metastasis (pM)
M0	No distant metastasis
M1	Distant metastasis
M1a	Non-regional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

1.4 Treatments for Prostate Cancer

The treatment options for prostate cancer vary in function and time of administration depending on the stage and degree of aggressiveness. The therapies also differ in terms of their complex effects on the body in combating cancer growth and spread not only at the observable/anatomical scale, but also at the molecular and biochemical levels. As such, there are always new studies and research projects aiming to develop and discover more appropriate and effective ways of combating cancer (Litwin & Tan, 2017). The main therapeutic options for prostate cancer include active surveillance, surgery, radiation therapy, hormone therapy, chemotherapy, and immunotherapy (Litwin & Tan, 2017). Discussing some of these treatment methods is vital in ultimately understanding the biochemical and genetic changes that cause alterations in important receptor molecules such as androgen receptor (AR) and glucocorticoid receptor (GR), subsequently leading to tumor evasion (Arora et al., 2013).

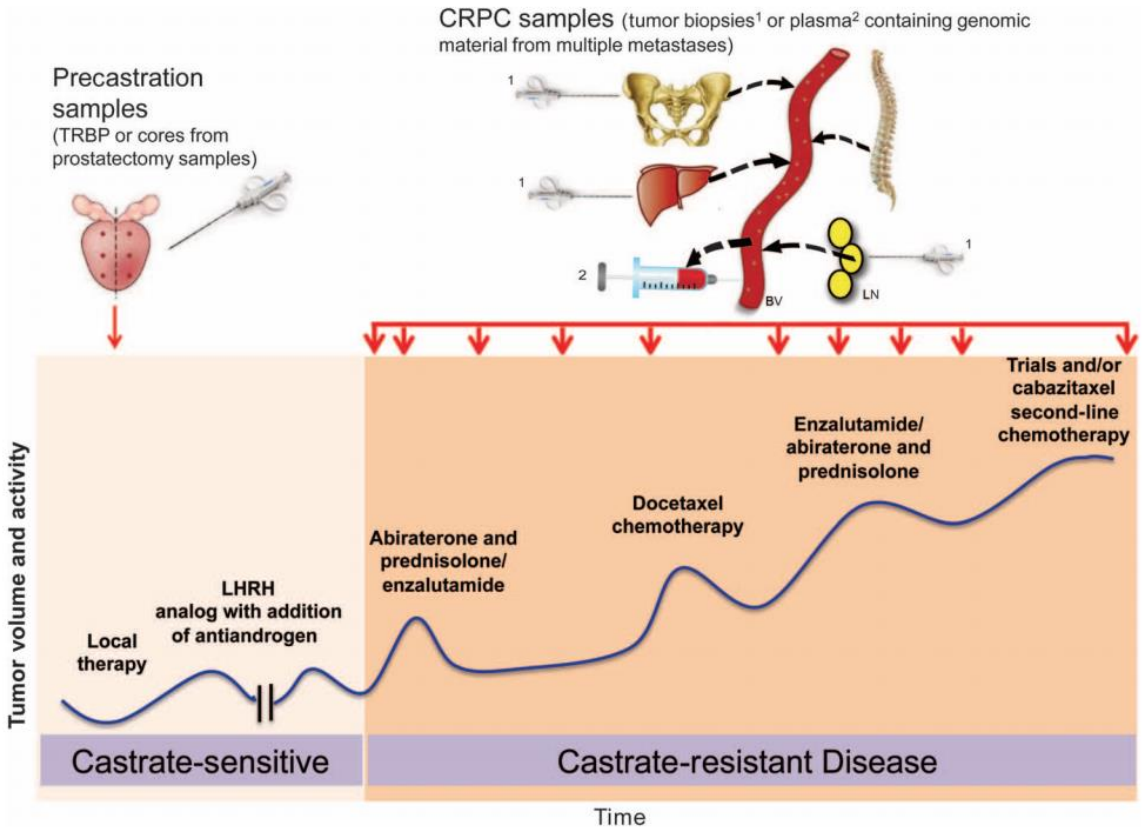


Figure 3: **Treatment Landscape in Prostate Cancer**. Diagram representing the different treatment options over the course of prostate cancer progression from local disease to metastatic (Carreira et al., 2014).

Several surgical approaches have been useful for managing local prostate cancer. These include radical prostatectomy, transurethral resection of the prostate gland, and orchiectomy. Radical prostatectomies are regularly performed in the advent of early stage prostate cancer because the removal of the entire prostate gland can temporarily reduce the effects of cancer and lead to better outcomes (Wilt et al., 2012). In many cases, prostate cancer can recur, but the procedure itself has been shown to prolong survival. A study showed that those patients receiving a radical prostatectomy had a 5.8% mortality rate compared to patients who underwent observation for localized prostate cancer, with

an 8.4% morality rate. There was also a significant reduction in all cause morality among men with PSA value greater than 10 ng/mL and who received a radical prostatectomy (Wilt et al., 2012). Transurethral resection of the prostate is a procedure used to remove a section of the gland (Pelletier et al., 2018). The last surgical approach is the least favorable because it involves the surgical castration of the patient by removal of the testes. Major psychological implications result due to the inability of the patient to maintain normal sexual function (Louda et al., 2012). Nevertheless, orchiectomy has significant benefits and is even comparable in terms of its positive outcomes on patients with cancer compared to other treatment options like radiotherapy. Orchiectomy may even have implications in the delay of metastatic prostate cancer (Fellows et al., 1992).

If prostate cancer progresses beyond medical intervention involving surgery or radiation, hormonal therapies can be an effective option for the patient. There are several different classes of hormone treatments including luteinizing hormone therapy (Lee, Kim, Choi, Lee, & Cho, 2018), CYP17 inhibitors (Ryan et al., 2013), and even anti-androgen drug therapies (Hussain et al., 2018). These classes differ in the biochemical pathways they target. These are effective agents for prostate cancer because the tumor cells seem to grow and spread rapidly by responding to the androgen production in the body. For example, testosterone and dihydrotestosterone (DHT) actually enhance the aggressiveness of the prostate cancer tumors (Nishiyama, Ikarashi, Hashimoto, Wako, & Takahashi, 2007). Luteinizing hormone agonists can decrease the production of androgens like testosterone in the body (Lee et al., 2018). Androgen drug therapies like enzalutamide can bind to intracellular receptors in the tumor cells and competitive block

androgen binding and subsequent translocation to the nucleus (Tran et al., 2009). Hormonal castration is usually a more favorable option for patients compared to surgical castration like orchiectomies (Anderson, Abrahamsson, Crawford, Miller, & Tombal, 2008). Therefore, the more common use of these treatments in practice requires an in-depth understanding of the pathways that are involved.

Studies have illustrated the prognostic significance of testosterone levels in patients with metastatic prostate cancer treated with luteinizing hormone-releasing hormone therapies (LH-RH). The more widely used LH-RH agonists for the maintenance, not treatment of prostate cancer, include leuprolide, triptorelin, and goserelin (Heyns et al., 2003; Yri, Bjoro, & Fossa, 2006). Common LH-RH agonists were used, and it was found that baseline testosterone values were very different from the 6-month testosterone readings (mean value at baseline was 440 ng/dL compared to 6-months 40 ng/dL). The decrease in testosterone levels indicate that the agonists have worked effectively (Perachino, Cavalli, & Bravi, 2010). Castrate levels are defined as testosterone <50 ng/dL (Klotz et al., 2015). A study showed that some men treated with leuprolide and goserelin reached castrate levels of testosterone after 3 months of being on the therapy (Yri et al., 2006). The use of leuprolide for instance targets the hypophyseal-gonadal axis. The downregulation of GnRH leads to inhibition of production for LH and FSH. As a result, testosterone production in the testes is suppressed (Singla, Ghandour, & Raj, 2019). LHRH treatments are very effective at reducing the testosterone levels and subsequently mitigating the aggressiveness of the tumor cells. However, this form of androgen

deprivation therapy (ADT) will ultimately be overcome and inevitably lead to castration resistant prostate cancer (CRPC) (Perner et al., 2015).

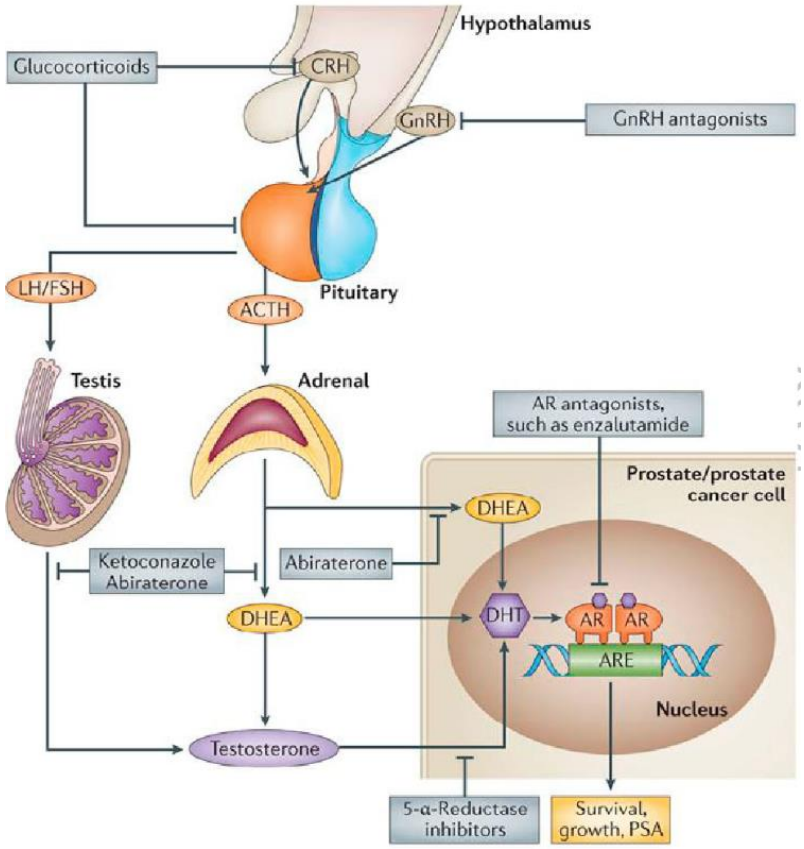


Figure 4: **Androgenic Synthesis Signaling Pathways and the Effects of Different Drugs.** Diagram representing LHRH antagonists disrupting the pathway that normally leads to the production of testosterone and DHT (Zhao, Urdaneta, & Anscher, 2016).

Abiraterone is a CYP17 inhibitor that acts by reducing testosterone levels by targeting mainly the adrenal tissue instead of the testes. The adrenal cortex also produces androgens such as testosterone that circulates throughout the body (Turcu, Smith, Auchus, & Rainey, 2014). Studies were conducted analyzing the effect of abiraterone in metastatic prostate cancer patients who did not have prior chemotherapy. Compared to

placebo, abiraterone plus prednisone revealed a 57% reducing in the risk of radiographic progression or death. Furthermore, patients had a 16.5 month of free survival compared to 8.3 months with prednisone alone (Ryan et al., 2013). In patients who had metastatic prostate cancer, the overall survival for the abiraterone-prednisone group was 14.8 months compared to the placebo-prednisone group at 10.9 months (de Bono et al., 2011). Abiraterone is an effective agent and this can be shown in prolonged survival of patients with metastatic prostate cancer who did not have prior chemotherapy and those that did.

The drug enzalutamide is a very common anti-androgen that is used alone or in combination with other types of therapies. There are several clinical studies involving enzalutamide in combination with other drugs like LHRH agonists, abiraterone, and even different forms of chemotherapy (Beer et al., 2014; Iguchi et al., 2019). Enzalutamide is used to restrict the aggressiveness of tumors presenting as metastatic castration-resistant prostate cancer (Kregel et al., 2016). It targets various nodes within the cancer cell in order to maximize its anti-androgen effects (Linder, van der Poel, Bergman, Zwart, & Prekovic, 2018). Enzalutamide was the main drug focused in our study, as well. We demonstrated the biochemical effects and changes that occur resulting from its application. One of the resistant mechanisms in mCRPC that involves enzalutamide is glucocorticoid receptor bypass (Arora et al., 2013). We present the need for novel combination therapies that may make enzalutamide a more potent inhibitor of AR signaling.

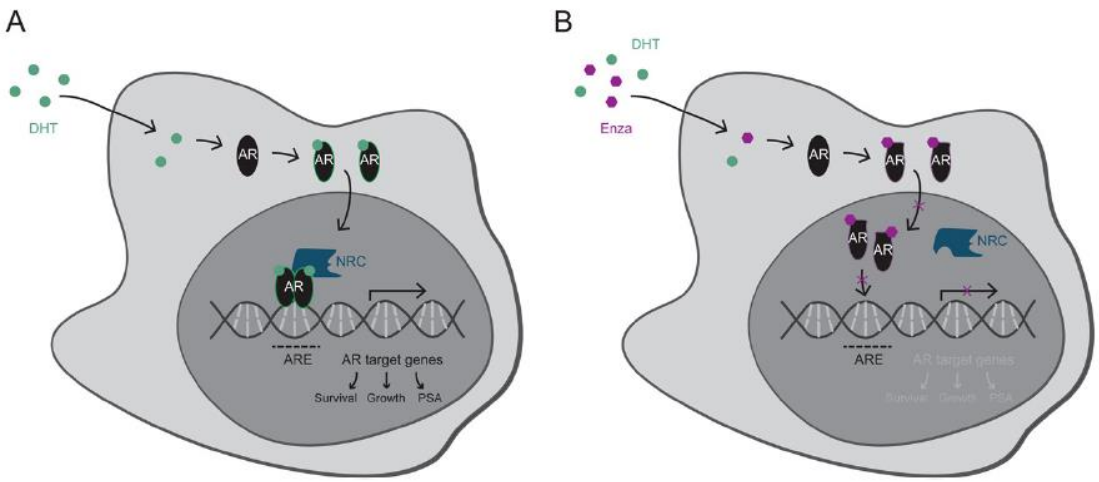


Figure 5: **AR Signaling Axis and Mechanism of Action of Enzalutamide.** This figure shows different nodes in the prostate cancer cell that are targeted by enzalutamide drug. The effects include binding to the AR protein, preventing translocation to specific genetic regions, and inhibiting expression of target genes (Linder et al., 2018).

As stated above, CRPC can persist if the tumor cells become resistant to ADT. Thus, the use of LHRH loses its effectiveness in slowing down progression. The requirement for alternative methods targeting tumor cells dependency on androgens is imperative. The use of enzalutamide and flutamide are drugs that meet these needs and are classified as anti-androgen drugs. In a study conducted using enzalutamide on patients who had nonmetastatic, castration resistant prostate cancer, treatment with the drug led to a 71% lower risk of metastasis compared to placebo (Hussain et al., 2018). These drugs can also be used in the treatment for metastatic castration resistant prostate cancer (mCRPC). Groups with metastatic prostate cancer treated with enzalutamide (n=872) compared to placebo (n=845) had significant differences for many important secondary end points including median time until initiation of cytotoxic chemotherapy,

median time until PSA progression, and even median time until first skeletal-related event (Beer et al., 2014). Patients who were administered enzalutamide (n=29) or flutamide (n=26), showed no significant difference in OS between the groups. Thus, the overall effectiveness of the drugs compared to each other is minimal and recognizing differences requires more specific analyses. For example, the same study found that the PSA-PFS was significantly longer in the enzalutamide group. Many adverse effects can occur with the use of these drugs and can include hypertension, liver dysfunction, and even diarrhea (Iguchi et al., 2019).

Finally, chemotherapy and immunotherapy can be treatment options for patients that have advanced metastatic prostate cancer. Common chemotherapeutic agents include docetaxel and cabazitaxel. Docetaxel given to patients who had advanced prostate cancer every three weeks had a median survival of 18.9 months compared to 17.4 months for the group that was given docetaxel weekly (Tannock et al., 2004). Administration of cabazitaxel was also found to be useful in targeting CRPC in patients who had prior docetaxel chemotherapy (C. J. Pezaro et al., 2014). Immunotherapy is gaining recognition as a potential area for targeting advanced prostate cancer. Harnessing the patient's immune system could lead to an alternative way of specifically targeting metastatic cancer cells. The only FDA drug for immunotherapy that is used to treat prostate cancer is sipuleucel-T. In a randomized, double-blind study, sipuleucel-T showed a 33% reduction in the risk of death (Higano et al., 2009). The median survival of patients on sipuleucel-T was 25.8 months compared to the placebo group 21.7 months (Kantoff et al., 2010).

1.5 Androgen Receptor and Glucocorticoid Receptor

The importance of understanding the treatment options lies in how they affect the molecular biology and the biochemistry of the patient at each stage of the cancer. Treatments can be divided based on how they affect the categories of castrate-sensitive prostate cancer (CSPC) as well as castrate-resistant prostate cancer (CRPC). CSPC specifies the tumor cells' dependency on the function of the androgen receptor (AR) (Hoang, Iczkowski, Kilari, See, & Nevalainen, 2017; Kohli et al., 2020). On the other hand, when the tumor cells become resistant to ADT, they progress into what is known as CRPC (Perner et al., 2015). Little is known about the biochemical pathways involved in the tumor evasion. Research has shown that there still is androgen dependency during CRPC, and that it may occur through different means such as AR mutations and amplifications (Taplin et al., 1999). However, the dependency for AR can be lost in this stage as the patient is taking enzalutamide (Kregel et al., 2016). As a result, there seems to be a compensatory mechanism involving glucocorticoid receptor (GR). That is, as AR is downregulated, GR seems to be upregulated in CRPC (Arora et al., 2013). As mentioned earlier, popular drugs for ADT include leuprolide and goserelin which are used during the CSPC stage, when there is androgen sensitivity. Therefore, their effects suggest that they are altering the regulation of AR and GR receptors (Sarosdy et al., 1998). Moreover, drug therapies involving enzalutamide may also facilitate these changes because they are common employed during CRPC, an androgen-insensitive stage (Kregel et al., 2016).

Androgen receptor is a very important biomolecule in prostate cancer. Its function ranges from DNA binding-dependent actions to non-DNA binding-dependent actions (Eder et al., 2001; Estrada, Espinosa, Muller, & Jaimovich, 2003). When the ligand is bound, in this case androgen molecules, the androgen/AR complex dimerizes and translocates to specific DNA binding regions (Koivisto, Kolmer, Visakorpi, & Kallioniemi, 1998). Attachment of the complex to the androgen response elements (AREs) on the DNA allows for specific targeting of genes and modulation of gene transcription. The androgen receptor responds to the ARE through its DNA binding domain (DBD) (Eder et al., 2001). The androgen receptor seems to have other mechanisms of action through non-genomic interactions. Given that the receptor molecule is located in the cytosol, convergence of different signals could occur through activation of 2nd messenger pathways including ERK, Akt, and MAPK (Kang et al., 2004; Kousteni et al., 2001).

Glucocorticoid receptor (GR) is another important molecule that is involved in prostate cancer. Current literature suggests it may play an important part in the understanding of how to target cancer spread and aggressiveness (Xie et al., 2015). When GR is not bound to a hormone such as cortisol, it is located in the cytosol of cells bound to other proteins including heat shock protein 90 and heat shock protein 70. GR can also be found complexed to FKBP5 (Pratt, Morishima, Murphy, & Harrell, 2006). When the ligand is bound to GR, there is homodimerization of the receptor and translocation to different regions of the nucleus. DNA responsive elements that complex with the receptor

protein assist in the recognition and transcription of various genes. The function of GR closely resembles that of AR (Buckingham, 2006).

Androgen deprivation therapy effectively slows down the progression of the prostate cancer by cutting off the cancer cells' response and dependency to hormones like DHT and testosterone (Heinlein & Chang, 2004). Prostate cancer cells utilize the AR protein to bind to androgens like testosterone in order to promote growth and survival (Gann, Hennekens, Ma, Longcope, & Stampfer, 1996). ADT has no curative properties for prostate cancer. It simply reduces the rate at which the cancer spreads, limits aggressiveness, and allows the urologist to effectively manage progression (Harris et al., 2009).

Ultimately however, the prostate cancer cells are able to bypass and evade the ADT treatment. At this point, the refractory stage of prostate cancer is castration resistant prostate cancer (CRPC) (Scher & Sawyers, 2005). Prostate cancer cells seem to become dependent on the AR protein again. What has been shown is an upregulation of the receptor protein. Treatments like enzalutamide are commonly used to create another androgen receptor blockade. Enzalutamide targets several different areas in the cell to mechanistically interfere with pathways at multiple points. These include competing with the androgens in binding to the AR protein, inhibiting AR nuclear translocation, and finally blocking DNA transactivation. Given that enzalutamide targets the AR pathway at multiple nodes, it has become a clinically very effective drug (Tran et al., 2009).

If patients do not respond to enzalutamide, their prostate cancer cells can become resistant to the drug. Research has discovered several potential mechanisms of resistance

to the drug (Claessens et al., 2014). First, there can be mutations with the AR protein receptor. Gain-of-function mutations have led to enhanced activity or upregulation of the AR gene. This was the drug was originally trying to counteract (Taplin et al., 1999). Next, AR splice variants can occur involving AR-V7. Post-transcriptional modification can induce alternatively spliced variants of AR. Isoforms like AR-V7 have been implicated in drug resistance to enzalutamide (Guo et al., 2009). Furthermore, GR has been shown to be upregulated after resistance to enzalutamide in an attempt by the prostate cancer cell to compensate for AR blockade. It has been hypothesized that GR takes over the role of AR at this stage, allowing the cells to thrive (Arora et al., 2013). Finally, prostate cancer cells can survive by utilizing a method of intratumoral androgen production (Locke et al., 2008).

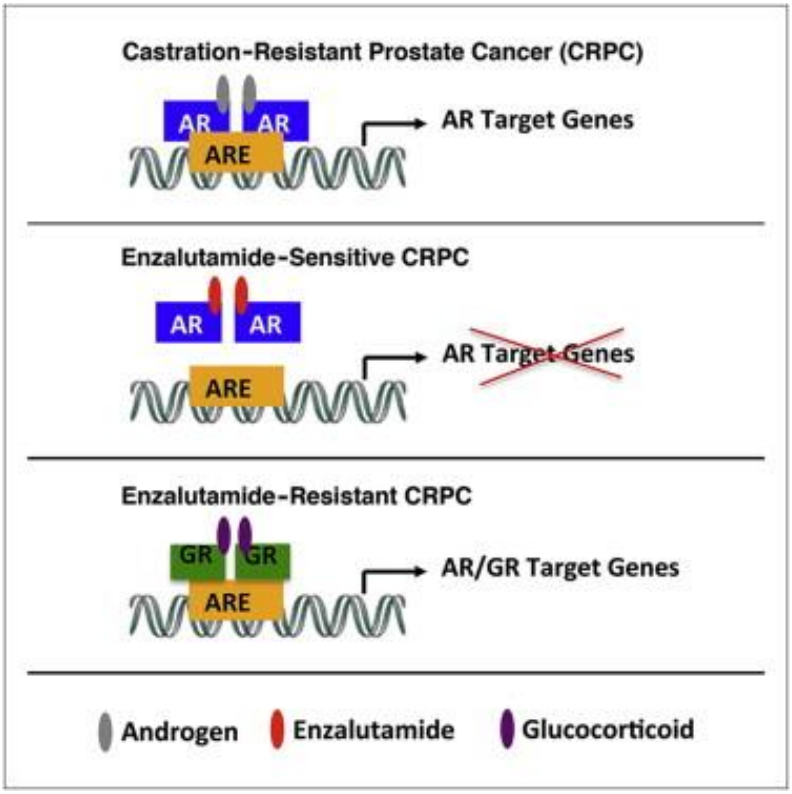


Figure 6: **Characterizing Gene Expression in Enzalutamide-Resistant Castrate Resistant Prostate Cancer**. This figure represents expression of AR and GR target genes during CRPC when AR protein is bound to either androgens or enzalutamide drug. Furthermore, during enzalutamide resistant CRPC, the GR protein takes over the function of AR (Arora et al., 2013).

There has been a keen focus on understanding how resistance to enzalutamide confers upregulation of the GR protein. Several drugs have been developed to completely block GR protein and thus once again interfere with cancer survival (Kach, Conzen, & Szmulewitz, 2015). However, the GR protein is located throughout the body in various tissue regions. Complete loss of the GR protein could have severely dangerous implications. Therefore, research has focused heavily on elucidating the specific effects associated with enzalutamide (Oakley & Cidlowski, 2013).

Upregulation of GR has been characterized as a method of enzalutamide resistance in prostate cancer by various means. Resistant cells show an upregulation of GR genes, with a simultaneous repression of androgen induced genes (Arora et al., 2013). In enzalutamide resistance cells (LREX), knockdown of GR led to a decrease in tumor growth. GR expressing resistant tumors showed an uneven restoration of AR target genes and even drove AR expression. This suggests that AR and GR had overlapping transcriptomes as GR function and transcriptomic action was able to compensate for loss of AR. Finally, GR expression was clearly shown to confer enzalutamide resistance through analysis of VCaP cells. VCaP cells are enzalutamide-sensitive cells, meaning that treatment of the drug can negative impact and reduce their growth. Treatment with dexamethasone induced upregulation of GR, conferring resistance of these cell lines.

However, when an antagonist to GR was presented, the sensitivity to enzalutamide was restored (Arora et al., 2013).

In other studies, several important characteristics about how enzalutamide resistant cells function in relation to GR have been elucidated. Using different genomic techniques, it was found that there is a specific GR enhancer that plays a major functional role in the resistance in CRPC. It is located on the H3K4me1track within the NR3C1 locus. Removal of the entire GR enhancer led to a 60% reduction in enzalutamide induced GR expression. Thus, excision of the enhancer mitigated GR function. This was an important finding because this specific GR enhancer was very unique to the prostate cancer tumor cells as opposed to being located in other types of tissues throughout the body (Shah et al., 2017).

Moreover, it was found that EZH2 and AR played very important roles in regulating the expression of GR genes (Shah et al., 2017). EZH2 is a gene that plays a role in DNA methylation. Specifically, it induces an epigenetic modification at H3K4me3 (Rosenfeld et al., 2009). H3K4me3 is involved in the reduction of GR expression (Caglio, Torlai Triglia, & Pombo, 2017). Under normal circumstances, enzalutamide drug promotes the activity of H3K4me3. However, H3K4me3 was not seen in enzalutamide resistant prostate cell lines which suggests that when the cells do not respond to the drug anymore, the cells do not express H3K4me3. Thus, GR expression is very high. Similarly, AR binding at the GR locus reduces GR expression. The research also discovered that BET inhibition can be used to re-sensitize the tumor cells to

enzalutamide drug by impairing the GR expression. BET proteins help to drive the expression of certain tissue specific genes (Shah et al., 2017).

Given the research and multiple analyses implicating the importance of GR in enzalutamide resistance, novel drugs have been created to further attempt to counteract cancer growth. GR antagonists such as arylpyrazole, agents targeting downstream AR targets, EZH2 inhibitors, and even inhibitors to the PI3K/AKT pathway have showed promising results (Arora et al., 2013; Bohrer, Chen, Hallstrom, & Huang, 2010; Carver et al., 2011; Vander Ark, Cao, & Li, 2018).

CHAPTER 2

OBJECTIVES

The aim of this project was to characterize GR expression and function and downstream targets in prostate cancer cells resistant to enzalutamide. Specifically, we wanted to understand whether or not resistance to enzalutamide drug could be reversed in these cell lines or if sensitivity to the drug could be re-established.

CHAPTER 3

METHODS

3.1 Overview

Multiple enzalutamide resistant prostate cancer cell line clones were developed in vitro in order to conduct this research project. In order to model enzalutamide resistance, we used prostate cancer LNCaP cell line. This is an androgen sensitive CRPC cell line that has intact androgen signaling (Horoszewicz et al., 1983; Lim et al., 1993). We looked at cell lines that were androgen sensitive and those that were androgen resistant for the purpose of characterizing GR and the downstream targets. Cell culture involved preparation of the specific colonies of interest. Western blots were used to analyze and characterize specific protein content in the cells. qPCR was used to amplify specific regions of DNA.

3.2 Cell Culture

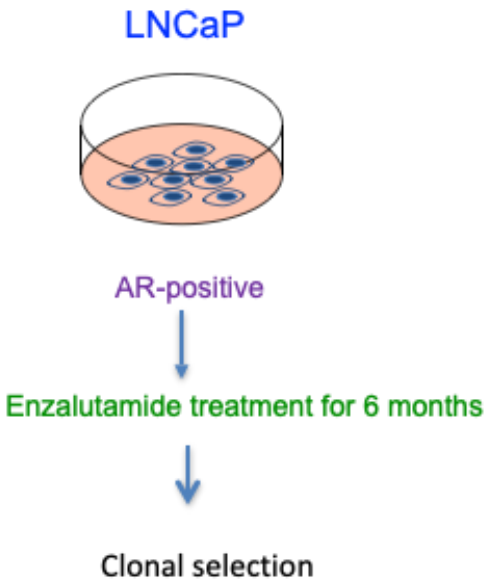


Figure 7: **Development of Enzalutamide Cell Lines.** LNCaP ATCC cells were grown in vitro. Treatment of enzalutamide to these AR sensitive cells occurred over the course of 6 months and allowed them to develop in response to the drug. Clonal selection was followed in order to begin the process of deeper investigative work and to characterize cellular response to enzalutamide.

The prostate cancer parental cell lines that we used for this study included LNCaP that were obtained from ATCC. The LNCaP cells are androgen-sensitive prostate cancer cell lines that were obtained from a left supraclavicular lymph node metastasis (Horoszewicz et al., 1983; Lim et al., 1993). LNCaP cells were cultured for several weeks in vitro. The cells were cultured with enzalutamide drug (10uM/ml of media) for about 6 months until there was clonal growth. Clonal selection of colonies occurred after treatment with enzalutamide drug. We expanded the clones that were resistant to enzalutamide treatment and characterized the clones. This was ongoing research in the laboratory.

3.3 Drugs/Chemicals

Different drugs were administered to these prostate cancer cell lines over the course of colony development. These drugs included enzalutamide as well as several GR inhibitors. Drugs were given in various doses and combinations for the same colonies. Preparation of enzalutamide involved dissolving and aliquoting it in media. Media consisted of 500 mL of RPMI, 10% FBS, and 5 mL of antimicrobial solution. GR inhibitors are associated with a company that does not want to disclose any information prior to a formal research publication.

3.4 Western Blot

Protein lysates for the western blot were prepared based on the cell lines we were interested in analyzing. The lysate solution used consisted of these chemicals. Centrifuging the lysates to separate protein supernatant from the cellular debris was performed. Lysate protein samples were divided up and labeled based on the various drug treatments and combinations used when preparing the colonies in vitro.

Protein estimation or quantification was then used to determine the total protein concentration for the sample lysates. Proper estimation was necessary to accurately determine the necessary amount of protein to load for each lane in the gel. In this case, a spectrophotometric technique measured the protein concentration sample based on specific absorbance patterns. Subsequent mathematical analysis involved normalizing the sample values, calculating the amount of 4x dye used, and determining the amount of lysis buffer to combine in each Eppendorf tube with their respective protein lysates. Protein estimation values were also compared to standard sample measurements as a benchmark for proper loading of the solutions.

In preparation for running the electrophoresis, we make 10% gels. The separating solutions consisted of dionized H20, 30% acrylamide mix, Tris-Cl (1.5 M, pH 8.8), 10% SDS, 10% ammonium persulfate, and TEMED (Bio-Rad Laboratories, Inc.). The stacking solution for the gel consisted of the same chemicals although Tris-Cl (1.0 M, pH 6.8) instead of Tris-Cl (1.5 M, pH 8.8) was used. The running buffer consisted of 25mM Tris-base, 192mM glycine, and 0.1% SDS (Bio-Rad Laboratories, Inc.). The gel was run at 133 volts for an hour.

Next, the transfer of the protein samples from the gels to the membrane was performed. The transfer buffer consisted of 25mM Tris-base, 192mM glycine, and nitrocellulose or 10% methanol. The transfer apparatus was operated at 73 volts for about an hour and a half.

Staining of the membrane involved shaking in ponceau solution to gauge the consistency of the bands as well as the band strength. The membranes were then placed in a 3% blocking solution consisting of TBST and milk powder. The blocking of the membrane took an hour. The TBST solution was also used for washing the membranes after blocking solution and probing for primary and secondary antibodies. TBST consists of Tris-Cl (pH 7.5), NaCl, deionized H20, and Tween (Bio-Rad Laboratories, Inc.). Thorough washes were primarily 3 times, 10 minutes each.

Primary antibodies and secondary antibodies were used for probing specific proteins of interest that were on the membrane. Antibodies were added to the 3% blocking solution at various dilutions depending on the strength and sensitivity of the antibody. Antibody targets were for AR and GR often in 1:2000 dilutions. The secondary antibody used for these protein targets was anti-rabbit. Primary antibodies were placed on the shaker for 1 hour and secondary antibodies were placed on the shaker for 1.5 hours at room temperature.

3.5 qPCR

For qPCR we used the Ssoadvanced universal SYBR green supermix kit (Bio-Rad Laboratories, Inc.). The components were initially thawed at room temperature prior to starting the experiment. We used a volume of 5 ul of the Ssoadvanced universal SYBR

green supermix at a 2x concentration per 10 ul reaction in order to obtain a final 1x concentration. Furthermore, the preparation involved 1 ul of the cDNA, 3 ul of water, and 1 ul of forward and reverse primers. The final volume of the reaction mix was 10 ul. All of the contents were thoroughly mixed to ensure homogeneity and were aliquoted into the PCR tubes (Bio-Rad Laboratories, Inc.).

The thermal cycling protocol was then performed on the CFX384 Touch real-time PCR system. Polymerase activation and amplification occurred at 95 degrees Celsius for 30 seconds and at 40 cycles. It was also done at 95 degrees Celsius for 15 seconds and at 60 cycles.

3.6 Statistical Analyses

For this experiment, we used the GraphPad statistics program to analyze the data gathered from the qPCR experiment. The qPCR experiment provided data on mRNA expression for different AR and GR targets. We ran multiple unpaired t-tests using the GraphPad software in order to look for significant differences between the mean values for the various AR and GR targets and their respective treatments (untreated, GR inhibitor 1, GR inhibitor 2). Significant p-values were represented at different levels including <0.05, <0.01, and <0.001.

CHAPTER 4

RESULTS

The microscopic images in Figure 8 showcase the effects of treatment with enzalutamide drug to LNCaP cell cultures. LNCaP cells that were treated with the drug had clear morphological changes, that were expected. Clumping of the cells and colony formation indicated in Figure 8b and 8c represent the characteristic cellular changes that occur in the process of developing resistance to the drug. The opposite can be seen in Figure 8a. LNCaP cells that were not treated with enzalutamide, but rather media alone, showed normal growth patterns.

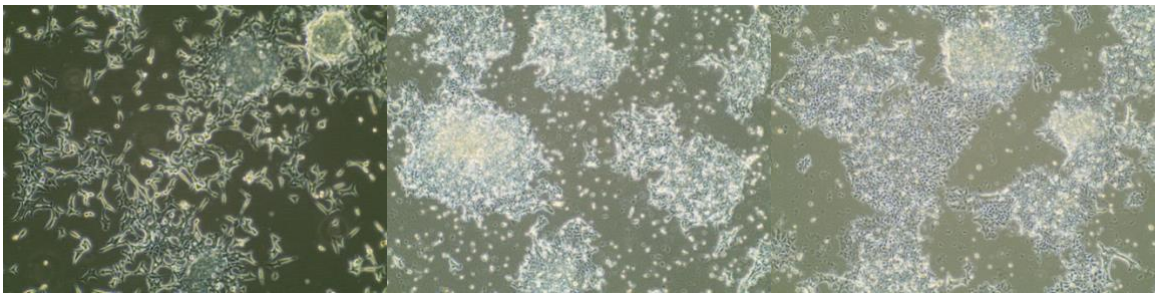
The western blot in Figure 9 illustrated the bands for actin that was used as an internal control. This indicates that the protein levels were correctly normalized across the different LNCaP cell lines.

The western blot diagram also illustrates stronger GR band signals for LnE-1,2,4 and 6 compared to LnE-8,A, and B. This suggests that enzalutamide resistance induced an upregulation of GR signaling in those selected clones. Alternatively, AR seems to be slightly downregulated in LnE-1,2,4, and 6 due to weaker band signals. Taken together, this information suggests that AR and GR may be linked and that a compensatory mechanism upregulates GR as AR is downregulated.

When analyzing LNCaP cells with no prior enzalutamide treatment, there was a band for the AR protein and an absence of a band for the GR protein. This result was to be expected because these cells are AR-positive cells that are not resistant to enzalutamide

and therefore are dependent on AR signaling. Furthermore, without prior treatment of the drug, we should not expect for there to be a high presence of GR.

Figure 10 indicates the AR targets that we screened in terms of relative mRNA expression. Relative mRNA expression levels of the AR targets were compared based on cells treated with either GR inhibitor 1 or 2, or untreated cells. Some of these targets were inhibited by GR inhibitors 1 and 2 compared to the untreated samples. These included AR, PSMA, PSA, and PAP. However, there were increases in relative mRNA expression for TRPM8, NKX3.1, and KLK2 after the GR inhibitors 1 and 2 compared to the untreated samples. Notably, there were significant decreases in mRNA expression for AR between untreated samples and those treated with GR inhibitor 1, $t(4)=12.33$, $p=0.0002$ as well as between untreated and treated with GR inhibitor 2, $t(4)=12.99$, $p=0.0002$. There were significant differences in mRNA expression for PSMA between untreated and treated with GR inhibitor 1 and 2, $t(4)=25.38$, $p=0.0001$ and, $t(4)=31.505$, $p=0.0001$, respectively. There were also significant differences in mRNA expression for PSA between untreated and those treated with GR inhibitor 1 and 2, $t(4)=5.47$, $p=0.0054$ and, $t(4)=13.47$, $p=0.0002$, respectively. Finally, there were significant decreases in mRNA expression for PAP between untreated and GR inhibitors 1 and 2, $t(4)=4.61$, $p=0.0099$ and , $t(4)=4.61$, $p=0.0099$, respectively. There were no significant differences in terms of mRNA expression between treatments for TRPM8, NKX3.1, and KLK2. Collectively, this data indicates that the treated cells had blocked AR signaling due to lower expression of AR, PSMA, PSA, and PAP. However, not all the targets are blocked by the inhibitors.



a. LNCaP-ATCC **b. LnE-Clone #2** **c. LnE-Clone A**

Figure 8: **Microscope images of model cell lines.** Microscopic images illustrating a. LNCaP ATCC cells with no treatment of enzalutamide drug compared to b-c. which consisted of different clone groups that were treated with enzalutamide drug. LNCaP-ATCC did not form major colonies or clusters which indicates that their growth and development persisted on media alone. LnE-Clone #2 and LnE-Clone A clearly indicates the development of colony formation. The subsistence of colonies during the course of drug treatment suggests that they are developing resistance to the drug.

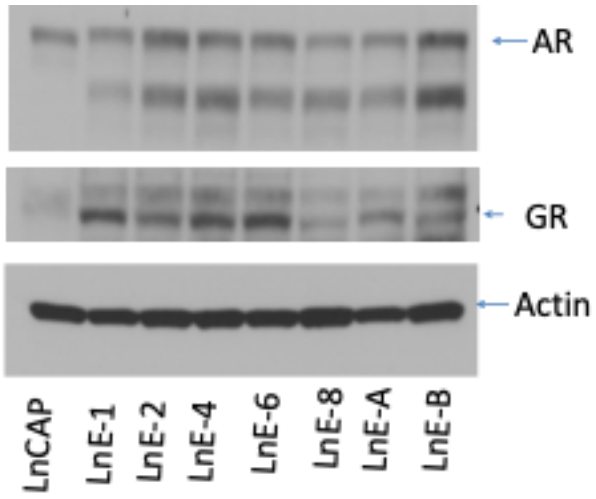


Figure 9: **Identifying molecular trends in model cell lines.** Western blot represents the LNCaP and enzalutamide resistant LnE clonal cell lines 1,2,4,6,8,A, and B. Actin served as an internal control. The presence or absence of AR and GR proteins are indicated by the relative strength of the band signals.

Figure 10: **GR and AR Targets.** This graph depicts various AR targets and their relative mRNA expression for untreated samples and those that were treated with two different GR inhibitors. There were substantial differences in mRNA expression between untreated samples versus GR inhibitors 1 and 2 for mRNA expression of PSA and PAP which are the key AR target genes. Significant p-values depicted in the graph were represented *<0.05, **<0.01, and ***<0.001.

CHAPTER 5

DISCUSSION

Current literature has suggested that during the metastatic stage of prostate cancer after the tumor cells have bypassed ADT, the AR protein may be upregulated again, and a newfound dependency of this signaling molecule occurs (Taplin et al., 1999). We have shown that this trend occurs by analyzing the western blot in Figure 9. In the LNCaP cells, the prostate cancer metastatic cells that are androgen-sensitive, the AR molecule has a strong signal and band strength (Lim et al., 1993). The GR molecule is notably absent as should be expected in this case because, in these cells, there is no AR blockade, and there is no need to bypass downregulation of AR with a compensatory mechanism (Lim et al., 1993).

However, while analyzing the other cell lines that were treated with the drug and eventually developed resistance to enzalutamide, there is a slightly different trend. In this case, some of the cell lines exhibit weak AR signals with a compensatory upregulation of the GR band signals. In this case, we have been able to mimic the enzalutamide resistance that occurs during the standard treatment of mCRPC.

Figure 10 illustrates the effect of GR inhibitors 1 and 2 on canonical AR targets. With the application of these inhibitors, AR, PSMA, PSA, and PAP have lower levels of mRNA expression compared to their untreated counterparts. The significance of this is that the cell's expressivity of these genetic elements is curbed in the advent that this potent inhibitor is applied. Moreover, this graph indicates that targeting GR is important as it seems to be involved in the expression of commonly known AR genes and target

elements. Astonishingly, there is a significant drop in expression levels of PSA and PAP from untreated samples. The presence and aggressiveness of prostate cancer cells have been measured using PSA levels and PAP levels. Higher levels, although not always accurate, indicate the presence of cancer (Catalona et al., 1994; Lin et al., 1998). The levels illustrated in figure 10 seem to suggest that the GR inhibitor reduces tumor burden. This is vitally important because this GR inhibitor could be used in combination with enzalutamide and create a more potent AR signaling blockade.

Similarly, there are reductions in the mRNA expression of AR and PSMA. AR has notoriously been implicated in tumor aggression and resistance. AR proteins respond to the androgen levels in the body by binding to DHT and testosterone. AR translocation to the nucleus enhances the growth and survival of the tumor cells (Koivisto et al., 1998). Reducing the targeted expression of AR suggests the importance of this drug as a targeted treatment. PSMA has also been shown to be associated with prostate cancer concerning its activation of the PI3K pathway (Caromile & Shapiro, 2017). Reductions in the expression of this protein molecule represent another way of targeting and reducing cancer burden. Specifically, GR inhibitor 2 rather than 1 induces a more significant reduction in AR, PSMA, PSA, and PAP, suggesting that it is more potent and effective compared to the other drug.

Figure 10 also indicates that GR inhibitors 1 and 2 cause an increase in mRNA expression of TRPM8, NKX3.1, and KLK2 compared to untreated conditions. TRPM8 is typically associated and elevated with androgen-dependent prostate cancer cells or androgen-sensitive cells like LNCaPs. Under normal conditions and with healthy

prostatic cells, its level is not largely expressed. TRPM8 has been implicated to have a protective role and seems to modulate the regulation of calcium ions during cancer invasiveness (Grolez & Gkika, 2016; Zhang & Barritt, 2006). Minor expression of the TRPM8 molecule in figure 10 could indicate that the GR inhibitors play a minor role in returning the cell to an androgen-dependent – like state.

NKX3.1 is a prostatic tumor suppressor gene that is almost completely lost in the majority of metastatic prostate cancers (Zhang & Barritt, 2006). However, here we see a significant expression of the gene compared to baseline with the administration of GR inhibitors. Effectively, the inhibitors are restoring the tumor suppressor activity that was initially lost. Furthermore, NKX3.1 is a shared AR and GR target. This suggests that GR is indeed functional.

The toxic effect of KLK2, being a genetic element that has cellular properties conducive to tumorigenesis, means that higher expression of this AR target is not beneficial in mitigating tumor burden (Shang et al., 2014). This is exactly what is seen when the GR inhibitors are used as a treatment. Although there are no clear functions of KLK2, it is typically associated with lower apoptosis and higher cell proliferation (Shang et al., 2014).

The protein patterns seen in the western blot, as well as the mRNA expression levels seen in the qPCR data, have characterized various target elements and pathways involved in AR signaling for enzalutamide resistant cells. The knockdown of the AR protein and the newfound dependency of GR protein in maintaining AR signaling suggest complicated overlapping pathways and the potential need for new combination therapies.

This research elucidates some of the intricate signaling pathways that involve AR target elements and the potential of GR inhibitors in restricting cancer growth and burden.

CHAPTER 6

CONCLUSION

During the metastatic setting of prostate cancer, when patients are given the first line of therapy, which is ADT, there is an improvement in the disease. Still, life expectancy only improves for a couple of months (Harris et al., 2009). What happens is that patients eventually develop resistance to this drug and stop responding to the medication. Then the tumor cells become even more aggressive. So, at that point, there is an increased need to find other therapies or combination therapies that would improve the sensitivity to enzalutamide (Scher & Sawyers, 2005). Active studies are being done to understand these problems, and researchers have used different model systems to understand what is going on (Scher & Sawyers, 2005). For example, this can be seen in the characterization of the biology or the signaling, and if there are drugs that can be proposed as combination therapies (Litwin & Tan, 2017). In our lab, we wanted to understand how enzalutamide resistance can be reversed or if we could restore the sensitivity to the drug. To do that, we had to develop cell lines and look at different signaling markers to understand what is targetable and to see the signaling that is functional in these cells. Existing literature suggests that there is an increase in GR in these cell lines (Arora et al., 2013). So, this means that GR is an excellent mechanism to bypass resistance and targeting that could be very relevant. In characterizing GR and the downstream targets, we found that both GR and GR signaling is present in these cell lines. Furthermore, using qPCR to measure mRNA expression of different AR and GR targets suggests that novel GR inhibitors 1 and 2 may prove to be useful in combating

cancer growth. Targeting GR could be a good option either alone or in combination with enzalutamide to increase drug sensitivity for tumor growth control during the metastatic setting.

REFERENCES

Anderson, J., Abrahamsson, P. A., Crawford, D., Miller, K., & Tombal, B. (2008). Management of advanced prostate cancer: can we improve on androgen deprivation therapy? *BJU International, 101*(12), 1497-1501. doi:10.1111/j.1464-410X.2008.07590.x

Armstrong, A. J., Anand, A., Edenbrandt, L., Bondesson, E., Bjartell, A., Widmark, A., . . . Morris, M. J. (2018). Phase 3 Assessment of the Automated Bone Scan Index as a Prognostic Imaging Biomarker of Overall Survival in Men With Metastatic Castration-Resistant Prostate Cancer: A Secondary Analysis of a Randomized Clinical Trial. *JAMA Oncology, 4*(7), 944-951. doi:10.1001/jamaoncol.2018.1093

Arora, V. K., Schenkein, E., Murali, R., Subudhi, S. K., Wongvipat, J., Balbas, M. D., . . . Sawyers, C. L. (2013). Glucocorticoid receptor confers resistance to antiandrogens by bypassing androgen receptor blockade. *Cell, 155*(6), 1309-1322. doi:10.1016/j.cell.2013.11.012

Azab, S., Osama, A., & Rafaat, M. (2012). Does normalizing PSA after successful treatment of chronic prostatitis with high PSA value exclude prostatic biopsy? *Translational Andrology and Urology, 1*(3), 148-152.

Beer, T. M., Armstrong, A. J., Rathkopf, D. E., Loriot, Y., Sternberg, C. N., Higano, C. S., . . . Investigators, P. (2014). Enzalutamide in metastatic prostate cancer before chemotherapy. *New England Journal of Medicine, 371*(5), 424-433.

Billis, A., Freitas, L. L. L., Costa, L. B. E., Barreto, I. S., Magna, L. A., Matheus, W. E., & Ferreira, U. (2019). The TNM 8th edition: Validation of the proposal for organ - confined (pT2) prostate cancer. *International Braz J Urol, 45*(2), 229-36. doi:10.1590/S1677-5538.IBJU.2018.0338

Bohrer, L. R., Chen, S., Hallstrom, T. C., & Huang, H. (2010). Androgens suppress EZH2 expression via retinoblastoma (RB) and p130-dependent pathways: a potential mechanism of androgen-refractory progression of prostate cancer. *Endocrinology, 151*(11), 5136-5145. doi:10.1210/en.2010-0436

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians, 68*(6), 394-424. doi:10.3322/caac.21492

Buckingham, J. C. (2006). Glucocorticoids: exemplars of multi-tasking. *British Journal of Pharmacology, 147 Suppl 1,* S258-268. doi:10.1038/sj.bjp.0706456

Caglio, G., Torlai Triglia, E., & Pombo, A. (2017). Keep Them Close: PRC2 Poises Enhancer-Promoter Interactions at Anterior Neuronal Genes. *Cell Stem Cell, 20*(5), 573-575. doi:10.1016/j.stem.2017.04.006

Caromile, L. A., & Shapiro, L. H. (2017). PSMA redirects MAPK to PI3K-AKT signaling to promote prostate cancer progression. *Molecular & Cellular Oncology, 4*(4), e1321168. doi:10.1080/23723556.2017.1321168

Carreira, S., Romanel, A., Goodall, J., Grist, E., Ferraldeschi, R., Miranda, S., . . . Attard, G. (2014). Tumor clone dynamics in lethal prostate cancer. *Science Translational Medicine, 6*(254), 254ra125. doi:10.1126/scitranslmed.3009448

Carver, B. S., Chapinski, C., Wongvipat, J., Hieronymus, H., Chen, Y., Chandarlapaty, S., . . . Sawyers, C. L. (2011). Reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient prostate cancer. *Cancer Cell, 19*(5), 575-586. doi:10.1016/j.ccr.2011.04.008

Catalona, W. J., Richie, J. P., Ahmann, F. R., Hudson, M. A., Scardino, P. T., Flanigan, R. C., . . . Southwick, P. C. (1994). Comparison of digital rectal examination and serum prostate specific antigen in the early detection of prostate cancer: results of a multicenter clinical trial of 6,630 men. *Journal of Urology, 151*(5), 1283-1290. doi:10.1016/s0022-5347(17)35233-3

Catalona, W. J., Smith, D. S., Ratliff, T. L., & Basler, J. W. (1993). Detection of organ-confined prostate cancer is increased through prostate-specific antigen-based screening. *JAMA: The Journal of the American Medical Association*, *270*(8), 948-954. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/7688438

Center, M. M., Jemal, A., Lortet-Tieulent, J., Ward, E., Ferlay, J., Brawley, O., & Bray, F. (2012). International variation in prostate cancer incidence and mortality rates. *European Urology, 61*(6), 1079-1092. doi:10.1016/j.eururo.2012.02.054

Chen, J., Wang, Z., Zhao, J., Zhu, S., Sun, G., Liu, J., . . . Zeng, H. (2019). Pelvic lymph node dissection and its extent on survival benefit in prostate cancer patients with a risk of lymph node invasion >5%: a propensity score matching analysis from SEER database. *Scientific Reports*, *9*(1), 17985. doi:10.1038/s41598-019-54261-4

Claessens, F., Helsen, C., Prekovic, S., Van den Broeck, T., Spans, L., Van Poppel, H., & Joniau, S. (2014). Emerging mechanisms of enzalutamide resistance in prostate cancer. *Nature Reviews. Urology, 11*(12), 712-716. doi:10.1038/nrurol.2014.243

Cohen, P., Peehl, D. M., Graves, H. C., & Rosenfeld, R. G. (1994). Biological effects of prostate specific antigen as an insulin-like growth factor binding protein-3 protease. *Journal of Endocrinology*, *142*(3), 407-415. doi:10.1677/joe.0.1420407

Cohen, R. J., Li, J., & Shannon, T. (2016). Prediction of bladder neck invasion and tumor extension to bladder neck margin by prostatic adenocarcinoma: a nomogram using biopsy data including transition zone tumor morphology. *Human Pathology, 57*, 85-90. doi:10.1016/j.humpath.2016.07.009

Cosma, G., Acampora, G., Brown, D., Rees, R. C., Khan, M., & Pockley, A. G. (2016). Prediction of Pathological Stage in Patients with Prostate Cancer: A Neuro-Fuzzy Model. *PLoS One, 11*(6), e0155856. doi:10.1371/journal.pone.0155856

Costanzo-Garvey, D. L., Keeley, T., Case, A. J., Watson, G. F., Alsamraae, M., Yu, Y., . . . Cook, L. M. (2020). Neutrophils are mediators of metastatic prostate cancer progression in bone. *Cancer Immunology, Immunotherapy*. doi:10.1007/s00262-020-02527-6

Dalton, D. L. (1989). Elevated serum prostate-specific antigen due to acute bacterial prostatitis. *Urology, 33*(6), 465. doi:10.1016/0090-4295(89)90131-3

de Bono, J. S., Logothetis, C. J., Molina, A., Fizazi, K., North, S., Chu, L., . . . Investigators, C.-A.-. (2011). Abiraterone and increased survival in metastatic prostate cancer. *New England Journal of Medicine, 364*(21), 1995-2005. doi:10.1056/NEJMoa1014618

de Lamirande, E. (2007). Semenogelin, the main protein of the human semen coagulum, regulates sperm function. *Seminars in Thrombosis and Hemostasis, 33*(1), 60-68. doi:10.1055/s-2006-958463

Eder, I. E., Culig, Z., Putz, T., Nessler-Menardi, C., Bartsch, G., & Klocker, H. (2001). Molecular biology of the androgen receptor: from molecular understanding to the clinic. *European Urology, 40*(3), 241-251. doi:10.1159/000049782

Estrada, M., Espinosa, A., Muller, M., & Jaimovich, E. (2003). Testosterone stimulates intracellular calcium release and mitogen-activated protein kinases via a G protein-coupled receptor in skeletal muscle cells. *Endocrinology, 144*(8), 3586-3597. doi:10.1210/en.2002-0164

Fellows, G. J., Clark, P. B., Beynon, L. L., Boreham, J., Keen, C., Parkinson, M. C., . . . Webb, J. N. (1992). Treatment of advanced localised prostatic cancer by orchiectomy, radiotherapy, or combined treatment. A Medical Research Council Study. Urological Cancer Working Party--Subgroup on Prostatic Cancer. *British Journal of Urology, 70*(3), 304-309. doi:10.1111/j.1464-410x.1992.tb15736.x

Gann, P. H., Hennekens, C. H., Ma, J., Longcope, C., & Stampfer, M. J. (1996). Prospective study of sex hormone levels and risk of prostate cancer. *Journal of the National Cancer Institute, 88*(16), 1118-1126. doi:10.1093/jnci/88.16.1118

Gleason, D. F., & Mellinger, G. T. (1974). Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. *Journal of Urology, 111*(1), 58-64. doi:10.1016/s0022-5347(17)59889-4

Grolez, G. P., & Gkika, D. (2016). TRPM8 Puts the Chill on Prostate Cancer. *Pharmaceuticals (Basel), 9*(3). doi:10.3390/ph9030044

Guo, Z., Yang, X., Sun, F., Jiang, R., Linn, D. E., Chen, H., . . . Qiu, Y. (2009). A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth. *Cancer Research, 69*(6), 2305-2313. doi:10.1158/0008-5472.CAN-08-3795

Hague, C., O'Connor, L. P., Wang, A. Z., Gomella, P. T., Yerram, N. K., Merino, M. J., & Pinto, P. A. (2020). MRI-guided pelvic lymph node biopsy via transrectal approach in prostate cancer. *Urology Case Reports, 30*, 101129. doi:10.1016/j.eucr.2020.101129

Harris, W. P., Mostaghel, E. A., Nelson, P. S., & Montgomery, B. (2009). Androgen deprivation therapy: progress in understanding mechanisms of resistance and optimizing androgen depletion. *Nature Clinical Practice. Urology, 6*(2), 76-85. doi:10.1038/ncpuro1296

Heinlein, C. A., & Chang, C. (2004). Androgen receptor in prostate cancer. *Endocrine Reviews, 25*(2), 276-308. doi:10.1210/er.2002-0032

Heyns, C. F., Simonin, M. P., Grosgurin, P., Schall, R., Porchet, H. C., & South African Triptorelin Study, G. (2003). Comparative efficacy of triptorelin pamoate and leuprolide acetate in men with advanced prostate cancer. *BJU International, 92*(3), 226-231. doi:10.1046/j.1464-410x.2003.04308.x

Higano, C. S., Schellhammer, P. F., Small, E. J., Burch, P. A., Nemunaitis, J., Yuh, L., . . . Frohlich, M. W. (2009). Integrated data from 2 randomized, double-blind, placebo-controlled, phase 3 trials of active cellular immunotherapy with sipuleucel-T in advanced prostate cancer. *Cancer, 115*(16), 3670-3679. doi:10.1002/cncr.24429

Hoang, D. T., Iczkowski, K. A., Kilari, D., See, W., & Nevalainen, M. T. (2017). Androgen receptor-dependent and -independent mechanisms driving prostate cancer progression: Opportunities for therapeutic targeting from multiple angles. *Oncotarget, 8*(2), 3724-3745. doi:10.18632/oncotarget.12554

Horoszewicz, J. S., Leong, S. S., Kawinski, E., Karr, J. P., Rosenthal, H., Chu, T. M., . . . Murphy, G. P. (1983). LNCaP model of human prostatic carcinoma. *Cancer Research, 43*(4), 1809-1818. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/6831420

Hussain, M., Fizazi, K., Saad, F., Rathenborg, P., Shore, N., Ferreira, U., . . . Sternberg, C. N. (2018). Enzalutamide in Men with Nonmetastatic, Castration-Resistant Prostate Cancer. *New England Journal of Medicine, 378*(26), 2465-2474. doi:10.1056/NEJMoa1800536

Iguchi, T., Tamada, S., Kato, M., Yasuda, S., Otoshi, T., Hamada, K., . . . Nakatani, T. (2019). Enzalutamide versus flutamide for castration-resistant prostate cancer after combined androgen blockade therapy with bicalutamide: a retrospective study. *International Journal of Clinical Oncology, 24*(7), 848-856.

Incerti, E., Mapelli, P., Gianolli, L., & Picchio, M. (2017). PET imaging for lymph node dissection in prostate cancer. *World Journal of Urology, 35*(4), 507-515. doi: 10.1007/s00345-016-1954-8

Kach, J., Conzen, S. D., & Szmulewitz, R. Z. (2015). Targeting the glucocorticoid receptor in breast and prostate cancers. *Science Translational Medicine, 7*(305), 305ps319. doi:10.1126/scitranslmed.aac7531

Kang, H. Y., Cho, C. L., Huang, K. L., Wang, J. C., Hu, Y. C., Lin, H. K., . . . Huang, K. E. (2004). Nongenomic androgen activation of phosphatidylinositol 3-kinase/Akt signaling pathway in MC3T3-E1 osteoblasts. *Journal of Bone and Mineral Research, 19*(7), 1181-1190. doi:10.1359/JBMR.040306

Kantoff, P. W., Higano, C. S., Shore, N. D., Berger, E. R., Small, E. J., Penson, D. F., . . . Investigators, I. S. (2010). Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *New England Journal of Medicine, 363*(5), 411-422.

Klotz, L., O'Callaghan, C., Ding, K., Toren, P., Dearnaley, D., Higano, C. S., . . . Crook, J. M. (2015). Nadir testosterone within first year of androgen-deprivation therapy (ADT) predicts for time to castration-resistant progression: a secondary analysis of the PR-7 trial of intermittent versus continuous ADT. *Journal of Clinical Oncology, 33*(10), 1151-1156. doi:10.1200/JCO.2014.58.2973

Kohli, M., Tan, W., Zheng, T., Wang, A., Montesinos, C., Wong, C., . . . Azad, A. A. (2020). Clinical and genomic insights into circulating tumor DNA-based alterations across the spectrum of metastatic hormone-sensitive and castrate-resistant prostate cancer. *EBioMedicine, 54*, 102728. doi:10.1016/j.ebiom.2020.102728

Koivisto, P., Kolmer, M., Visakorpi, T., & Kallioniemi, O. P. (1998). Androgen receptor gene and hormonal therapy failure of prostate cancer. *American Journal of Pathology*, *152*(1), 1-9.

Kousteni, S., Bellido, T., Plotkin, L. I., O'Brien, C. A., Bodenner, D. L., Han, L., . . . Manolagas, S. C. (2001). Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell, 104*(5), 719-730. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11257226

Kregel, S., Chen, J. L., Tom, W., Krishnan, V., Kach, J., Brechka, H., . . . Vander Griend, D. J. (2016). Acquired resistance to the second-generation androgen receptor antagonist enzalutamide in castration-resistant prostate cancer. *Oncotarget, 7*(18), 26259-26274. doi:10.18632/oncotarget.8456

Kumar, A., Mikolajczyk, S. D., Goel, A. S., Millar, L. S., & Saedi, M. S. (1997). Expression of pro form of prostate-specific antigen by mammalian cells and its conversion to mature, active form by human kallikrein 2. *Cancer Research, 57*(15), 3111-14. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9242434

Lee, D. S., Kim, S. J., Choi, G. W., Lee, Y. B., & Cho, H. Y. (2018). Pharmacokinetic-Pharmacodynamic Model for the Testosterone-Suppressive Effect of Leuprolide in Normal and Prostate Cancer Rats. *Molecules*, *23*(4). doi:10.3390/molecules23040909

Lim, D. J., Liu, X. L., Sutkowski, D. M., Braun, E. J., Lee, C., & Kozlowski, J. M. (1993). Growth of an androgen-sensitive human prostate cancer cell line, LNCaP, in nude mice. *Prostate*, *22*(2), 109-118. doi:10.1002/pros.2990220203

Lin, M. F., Meng, T. C., Rao, P. S., Chang, C., Schonthal, A. H., & Lin, F. F. (1998). Expression of human prostatic acid phosphatase correlates with androgen-stimulated cell proliferation in prostate cancer cell lines. *Journal of Biological Chemistry*, *273*(10), 5939-5947. doi:10.1074/jbc.273.10.5939

Linder, S., van der Poel, H. G., Bergman, A. M., Zwart, W., & Prekovic, S. (2018). Enzalutamide therapy for advanced prostate cancer: efficacy, resistance and beyond. *Endocrine-Related Cancer*, *26*(1), R31-R52. doi:10.1530/ERC-18-0289

Litwin, M. S., & Tan, H. J. (2017). The Diagnosis and Treatment of Prostate Cancer: A Review. *JAMA: The Journal of the American Medical Assn*, *317*(24), 2532-2542.

Locke, J. A., Guns, E. S., Lubik, A. A., Adomat, H. H., Hendy, S. C., Wood, C. A., . . . Nelson, C. C. (2008). Androgen levels increase by intratumoral de novo

steroidogenesis during progression of castration-resistant prostate cancer. *Cancer Research, 68*(15), 6407-6415. doi:10.1158/0008-5472.CAN-07-5997

Louda, M., Valis, M., Splichalova, J., Pacovsky, J., Khaled, B., Podhola, M., . . . Brodak, M. (2012). Psychosocial implications and the duality of life outcomes for patients with prostate carcinoma after bilateral orchiectomy. *Neuro Endocrinology Letters, 33*(8), 761-764. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/23391980

Lovgren, J., Valtonen-Andre, C., Marsal, K., Lilja, H., & Lundwall, A. (1999). Measurement of prostate-specific antigen and human glandular kallikrein 2 in different body fluids. *Journal of Andrology, 20*(3), 348-355. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10386814

Lundwall, A., & Lilja, H. (1987). Molecular cloning of human prostate specific antigen cDNA. *FEBS Letters, 214*(2), 317-322. doi:10.1016/0014-5793(87)80078-9

Mohler, J. L., Armstrong, A. J., Bahnson, R. R., D'Amico, A. V., Davis, B. J., Eastham, J. A., . . . Freedman-Cass, D. A. (2016). Prostate Cancer, Version 1.2016. *Journal of the National Comprehensive Cancer Network, 14*(1), 19-30.

Nishiyama, T., Ikarashi, T., Hashimoto, Y., Wako, K., & Takahashi, K. (2007). The change in the dihydrotestosterone level in the prostate before and after androgen deprivation therapy in connection with prostate cancer aggressiveness using the Gleason score. *Journal of Urology, 178*(4 Pt 1), 1282-1288; discussion 1288-289. doi:10.1016/j.juro.2007.05.138

Oakley, R. H., & Cidlowski, J. A. (2013). The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease. *Journal of Allergy and Clinical Immunology, 132*(5), 1033-1044. doi:10.1016/j.jaci.2013.09.007

Panach-Navarrete, J., Garcia-Morata, F., Hernandez-Medina, J. A., & Martinez-Jabaloyas, J. M. (2015). When to biopsy seminal vesicles. *Actas Urologicas Españolas, 39*(4), 203-209. doi:10.1016/j.acuro.2014.10.006

PDQ Adult Treatment Editorial Board. Prostate Cancer Treatment (PDQ®): Health Professional Version. 2020 Jan 29. In: PDQ Cancer Information Summaries [Internet]. Bethesda (MD): National Cancer Institute (US); 2002-. [Figure, Figure 1. Anatomy of the male reproductive and urinary systems.] Available from: https://www.ncbi.nlm.nih.gov/books/NBK66036/figure/CDR0000062910__1685/

Pelletier, J., Cyr, S. J., Julien, A. S., Fradet, Y., Lacombe, L., & Toren, P. (2018). Contemporary outcomes of palliative transurethral resection of the prostate in patients with locally advanced prostate cancer. *Urologic Oncology, 36*(8), 363 e367-363 e311. doi:10.1016/j.urolonc.2018.05.004

Perachino, M., Cavalli, V., & Bravi, F. (2010). Testosterone levels in patients with metastatic prostate cancer treated with luteinizing hormone-releasing hormone therapy: prognostic significance? *BJU International, 105*(5), 648-651. doi:10.1111/j.1464-410X.2009.08814.x

Perner, S., Cronauer, M. V., Schrader, A. J., Klocker, H., Culig, Z., & Baniahmad, A. (2015). Adaptive responses of androgen receptor signaling in castration-resistant prostate cancer. *Oncotarget, 6*(34), 35542-35555. doi:10.18632/oncotarget.4689

Pezaro, C., Woo, H. H., & Davis, I. D. (2014). Prostate cancer: measuring PSA. *Internal Medicine Journal, 44*(5), 433-440. doi:10.1111/imj.12407

Pezaro, C. J., Omlin, A. G., Altavilla, A., Lorente, D., Ferraldeschi, R., Bianchini, D., . . . Attard, G. (2014). Activity of cabazitaxel in castration-resistant prostate cancer progressing after docetaxel and next-generation endocrine agents. *European Urology, 66*(3), 459-465. doi:10.1016/j.eururo.2013.11.044

Porcaro, A. B., Corsi, P., Inverardi, D., Sebben, M., Tafuri, A., Processali, T., . . . Artibani, W. (2018). Prostate-specific antigen associates with extensive lymph node invasion in high-risk prostate cancer. *Tumori, 104*(4), 307-311. doi:10.1177/0300891618765567

Pratt, W. B., Morishima, Y., Murphy, M., & Harrell, M. (2006). Chaperoning of glucocorticoid receptors. *Handbook of Experimental Pharmacology* (172), 111-138. doi:10.1007/3-540-29717-0_5

Riegman, P. H., Vlietstra, R. J., van der Korput, J. A., Brinkmann, A. O., & Trapman, J. (1991). The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. *Molecular Endocrinology, 5*(12), 1921-1930. doi:10.1210/mend-5-12-1921

Rosenfeld, J. A., Wang, Z., Schones, D. E., Zhao, K., DeSalle, R., & Zhang, M. Q. (2009). Determination of enriched histone modifications in non-genic portions of the human genome. *BMC Genomics, 10*, 143. doi:10.1186/1471-2164-10-143

Ryan, C. J., Smith, M. R., de Bono, J. S., Molina, A., Logothetis, C. J., de Souza, P., . . . Investigators, C.-A.-. (2013). Abiraterone in metastatic prostate cancer without previous chemotherapy. *New England Journal of Medicine, 368*(2), 138-148. doi:10.1056/NEJMoa1209096

Sarosdy, M. F., Schellhammer, P. F., Sharifi, R., Block, N. L., Soloway, M. S., Venner, P. M., . . . Kolvenbag, G. J. (1998). Comparison of goserelin and leuprolide in

combined androgen blockade therapy. *Urology, 52*(1), 82-88. doi:10.1016/s0090-4295(98)00145-9

Scher, H. I., & Sawyers, C. L. (2005). Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. *Journal of Clinical Oncology, 23*(32), 8253-261. doi:10.1200/JCO.2005.03.4777

Shah, N., Wang, P., Wongvipat, J., Karthaus, W. R., Abida, W., Armenia, J., . . . Sawyers, C. L. (2017). Regulation of the glucocorticoid receptor via a BET-dependent enhancer drives antiandrogen resistance in prostate cancer. *Elife, 6*. doi:10.7554/eLife.27861

Shang, Z., Niu, Y., Cai, Q., Chen, J., Tian, J., Yeh, S., . . . Chang, C. (2014). Human kallikrein 2 (KLK2) promotes prostate cancer cell growth via function as a modulator to promote the ARA70-enhanced androgen receptor transactivation. *Tumour Biology, 35*(3), 1881-1890. doi:10.1007/s13277-013-1253-6

Siegel, R. L., Miller, K. D., & Jemal, A. (2019). Cancer statistics, 2019. *CA: A Cancer Journal for Clinicians, 69*(1), 7-34. doi:10.3322/caac.21551

Siegel, R. L., Miller, K. D., & Jemal, A. (2020). Cancer statistics, 2020. *CA: A Cancer Journal for Clinicians, 70*(1), 7-30. doi:10.3322/caac.21590

Simmons, M. N., Berglund, R. K., & Jones, J. S. (2011). A practical guide to prostate cancer diagnosis and management. *Cleveland Clinic Journal of Medicine, 78*(5), 321-331. doi:10.3949/ccjm.78a.10104

Singla, N., Ghandour, R. A., & Raj, G. V. (2019). Investigational luteinizing hormone releasing hormone (LHRH) agonists and other hormonal agents in early stage clinical trials for prostate cancer. *Expert Opinion on Investigational Drugs, 28*(3), 249-259. doi:10.1080/13543784.2019.1570130

Spencer, J. A., Chng, W. J., Hudson, E., Boon, A. P., & Whelan, P. (1998). Prostate specific antigen level and Gleason score in predicting the stage of newly diagnosed prostate cancer. *British Journal of Radiology, 71*(851), 1130-1135. doi:10.1259/bjr.71.851.10434906

Stamey, T. A., Yang, N., Hay, A. R., McNeal, J. E., Freiha, F. S., & Redwine, E. (1987). Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. *New England Journal of Medicine, 317*(15), 909-916.

Tang, T., Yang, Z., Zhang, D., Qu, J., Liu, G., & Zhang, S. (2017). Clinicopathological study of 9 cases of prostate cancer involving the rectal wall. *Diagnostic Pathology, 12*(1), 8. doi:10.1186/s13000-017-0599-2

Tannock, I. F., de Wit, R., Berry, W. R., Horti, J., Pluzanska, A., Chi, K. N., . . . Investigators, T. A. X. (2004). Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *New England Journal of Medicine, 351*(15), 1502-1512. doi:10.1056/NEJMoa040720

Taplin, M. E., Bubley, G. J., Ko, Y. J., Small, E. J., Upton, M., Rajeshkumar, B., & Balk, S. P. (1999). Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. *Cancer Research, 59*(11), 2511-2515. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10363963

Tarhan, F., Demir, K., Orcun, A., & Madenci, O. C. (2016). Effect of ejaculation on Serum Prostate-Specific Antigen concentration. *International Braz J Urol, 42*(3), 472-478. doi:10.1590/S1677-5538.IBJU.2015.0116

Thompson, I. M., Pauler, D. K., Goodman, P. J., Tangen, C. M., Lucia, M. S., Parnes, H. L., . . . Coltman, C. A., Jr. (2004). Prevalence of prostate cancer among men with a prostate-specific antigen level < or =4.0 ng per milliliter. *New England Journal of Medicine, 350*(22), 2239-2246. doi:10.1056/NEJMoa031918

Tran, C., Ouk, S., Clegg, N. J., Chen, Y., Watson, P. A., Arora, V., . . . Sawyers, C. L. (2009). Development of a second-generation antiandrogen for treatment of advanced prostate cancer. *Science, 324*(5928), 787-790. doi:10.1126/science.1168175

Turcu, A., Smith, J. M., Auchus, R., & Rainey, W. E. (2014). Adrenal androgens and androgen precursors-definition, synthesis, regulation and physiologic actions. *Comprehensive Physiology, 4*(4), 1369-1381. doi:10.1002/cphy.c140006

Umeda, T., Koizumi, M., Fukai, S., Miyaji, N., Motegi, K., Nakazawa, S., & Takiguchi, T. (2018). Evaluation of bone metastatic burden by bone SPECT/CT in metastatic prostate cancer patients: defining threshold value for total bone uptake and assessment in radium-223 treated patients. *Annals of Nuclear Medicine, 32*(2), 105-113. doi:10.1007/s12149-017-1224-x

Vander Ark, A., Cao, J., & Li, X. (2018). Mechanisms and Approaches for Overcoming Enzalutamide Resistance in Prostate Cancer. *Frontiers in Oncology, 8*, 180. doi:10.3389/fonc.2018.00180

Warner, W. A., Lee, T. Y., Badal, K., Williams, T. M., Bajracharya, S., Sundaram, V., . . . Llanos, A. A. M. (2018). Cancer incidence and mortality rates and trends in Trinidad and Tobago. *BMC Cancer, 18*(1), 712. doi:10.1186/s12885-018-4625-x

Wilt, T. J., Brawer, M. K., Jones, K. M., Barry, M. J., Aronson, W. J., Fox, S., . . . Prostate Cancer Intervention versus Observation Trial Study, G. (2012). Radical prostatectomy versus observation for localized prostate cancer. *New England Journal of Medicine, 367*(3), 203-213. doi:10.1056/NEJMoa1113162

Xie, N., Cheng, H., Lin, D., Liu, L., Yang, O., Jia, L., . . . Dong, X. (2015). The expression of glucocorticoid receptor is negatively regulated by active androgen receptor signaling in prostate tumors. *International Journal of Cancer, 136*(4), E27-38. doi:10.1002/ijc.29147

Yousef, G. M., & Diamandis, E. P. (2001). The new human tissue kallikrein gene family: structure, function, and association to disease. *Endocrine Reviews, 22*(2), 184-204. doi:10.1210/edrv.22.2.0424

Yri, O. E., Bjoro, T., & Fossa, S. D. (2006). Failure to achieve castration levels in patients using leuprolide acetate in locally advanced prostate cancer. *European Urology, 49*(1), 54-58; discussion 58. doi:10.1016/j.eururo.2005.09.009

Zang, L., Ma, M., Hu, J., Qiu, H., Huang, B., & Chu, T. (2015). The effects of lung and prostate cancer bone metastasis on serum osteoprotegerin levels: a meta-analysis. *Scientific Reports, 5*, 18324. doi:10.1038/srep18324

Zhang, L., & Barritt, G. J. (2006). TRPM8 in prostate cancer cells: a potential diagnostic and prognostic marker with a secretory function? *Endocrine-Related Cancer, 13*(1), 27-38. doi:10.1677/erc.1.01093

Zhao, S., Urdaneta, A. I., & Anscher, M. S. (2016). The role of androgen deprivation therapy plus radiation therapy in patients with non-metastatic prostate cancer. *Expert Review of Anticancer Therapy, 16*(9), 929-942. doi:10.1080/14737140.2016.1218279